



**POLITECHNIKA
GDAŃSKA**

The author of the doctoral dissertation: **Anoop Kallingal**
Scientific discipline: **Chemical Sciences**

DOCTORAL DISSERTATION

Title of doctoral dissertation (in English): *Mechanistic studies on selected novel anticancer compounds modulating the function of telomeric proteins*

Title of doctoral dissertation (in Polish): *Badania mechanizmów działania wybranych nowych związków przeciwnowotworowych modulujących funkcję białek telomerowych*

Supervisor	Auxiliary supervisor
<i>signature</i>	<i>signature</i>
prof. dr hab. inż. Maciej Bagiński	dr inż. Natalia Maciejewska

Gdańsk, 2026

STATEMENT

The author of the doctoral dissertation: Anoop Kallingal

I, the undersigned, declare that I am aware that in accordance with the provisions of Art. 27 (1) and (2) of the Act of 4th February 1994 on Copyright and Related Rights (Journal of Laws of 2021, item 1062), the university may use my doctoral dissertation entitled: "Mechanistic studies on selected novel anticancer compounds modulating the function of telomeric proteins" for scientific or didactic purposes.¹

Gdańsk,

.....
signature of the PhD student

Aware of criminal liability for violations of the Act of 4th February 1994 on Copyright and Related Rights and disciplinary actions set out in the Law on Higher Education and Science (Journal of Laws 2021, item 478), as well as civil liability, I declare, that the submitted doctoral dissertation is my own work.

I declare, that the submitted doctoral dissertation is my own work performed under and in cooperation with the supervision of prof. dr hab. inż. Maciej Bagiński, ~~the second supervision of <name of the second supervisor>~~, the auxiliary supervision of dr inż. Natalia Maciejewska, ~~the cosupervision of <name of the cosupervisor>~~.*.

This submitted doctoral dissertation has never before been the basis of an official procedure associated with the awarding of a PhD degree.

All the information contained in the above thesis which is derived from written and electronic sources is documented in a list of relevant literature in accordance with Art. 34 of the Copyright and Related Rights Act.

I confirm that this doctoral dissertation is identical to the attached electronic version.

Gdańsk,

.....
signature of the PhD student

I, the undersigned, agree/~~do not agree~~* to include an electronic version of the above doctoral dissertation in the open, institutional, digital repository of Gdańsk University of Technology.

Gdańsk,

.....
signature of the PhD student

¹ Art. 27. 1. Instytucje oświatowe oraz podmioty, o których mowa w art. 7 ust. 1 pkt 1, 2 i 4-8 ustawy z dnia 20 lipca 2018 r. - Prawo o szkolnictwie wyższym i nauce, mogą na potrzeby zilustrowania treści przekazywanych w celach dydaktycznych lub w celu prowadzenia działalności naukowej korzystać z rozpowszechnionych utworów w oryginale i w tłumaczeniu oraz zwielokrotniać w tym celu rozpowszechnione drobne utwory lub fragmenty większych utworów.

2. W przypadku publicznego udostępniania utworów w taki sposób, aby każdy mógł mieć do nich dostęp w miejscu i czasie przez siebie wybranym korzystanie, o którym mowa w ust. 1, jest dozwolone wyłącznie dla ograniczonego kręgu osób uczących się, nauczających lub prowadzących badania naukowe, zidentyfikowanych przez podmioty wymienione w ust. 1.

DESCRIPTION OF DOCTORAL DISSERTATION

The Author of the doctoral dissertation: Anoop Kallingal

Title of doctoral dissertation: Mechanistic studies on selected novel anticancer compounds modulating the function of telomeric proteins.

Title of doctoral dissertation in Polish: Badania mechanizmów działania wybranych nowych związków przeciwnowotworowych modulujących funkcję białek telomerowych.

Language of doctoral dissertation: English

Supervisor: prof. dr hab. inż. Maciej Bagiński

Auxiliary supervisor*: dr inż. Natalia Maciejewska

Date of doctoral defense:

Keywords of doctoral dissertation in Polish:

nowotwór, białka shelterinowe, telomer, związki przeciwnowotworowe

Keywords of doctoral dissertation in English:

cancer, shelterin proteins, telomere, anti-cancer compounds

Summary of doctoral dissertation in Polish:

Komórki nowotworowe potrzebują ochrony telomerów, aby utrzymać proliferację. Niniejsza rozprawa badała, czy A822 i jego nowe pochodne TRFiN mogą zaburzać oddziaływanie TRF1 (czynn timer 1 wiążący powtórzenia telomerowe) z TIN2 (czynn timer jądrowy 2 oddziałujący z TRF1) i ograniczać progresję komórek nowotworowych. Związki A822 i TRFiN analizowano za pomocą modelowania, powierzchniowego rezonansu plazmonowego, badań cytotoksyczności i testów funkcjonalnych w komórkach HeLa i MCF7. Aktywne pochodne, TRFiN41, TRFiN49 i TRFiN72, oddziaływały z domeną TRFH TRF1 i wywoływały efekty zgodne z zaburzeniami aktywności shelterin. Traktowanie komórek zwiększyło liczbę komórek γ H2AX-dodatnich, nasiliło kolokalizację TIN2- γ H2AX, zaburzyło progresję widełek replikacyjnych i wywołało nieprawidłowości telomerów bez znacznego skracania telomerów. Zmiany te skutkowały zaburzeniami cyklu komórkowego, zmniejszeniem proliferacji, starzeniem się komórek, uszkodzeniem mitochondriów i opóźnieniem apoptozy. Związki te hamowały również migrację, tworzenie kolonii, wzrost sferoidów, angiogenezę i wzrost kolonii komórek nowotworowych. Analiza transkryptomiczna wykazała supresję replikacji DNA i genów cyklu komórkowego, a także aktywację szlaków stresu i apoptozy. Odkrycia te potwierdzają, że nowe związki TRFiN wywodzące się z A822 działają poprzez stres telomerowy i replikacyjny, modulując funkcje białek shelterynowych, a nie poprzez natychmiastową, niespecyficzną toksyczność. TRFiN49 wykazał najsilniejszy profil i jest obiecującym związkiem wiodącym w terapii ukierunkowanej na telomery.

Summary of doctoral dissertation in English:

Cancer cells depend on telomere protection to sustain proliferation. This thesis examined whether A822 and its novel TRFiN derivatives can disturb the telomeric repeat binding factor 1 (TRF1) TRF1-interacting nuclear factor 2 (TIN2) axis and limit tumour cell progression. A822 and TRFiN compounds were analysed by molecular modelling, surface plasmon resonance, cytotoxicity screening and functional assays in HeLa and MCF7 cells. The active derivatives, TRFiN41, TRFiN49, and TRFiN72, interacted with the TRF1-TRFH domain and produced effects consistent with shelterin disruption. Treatment increased phosphorylated histone H2AX (γ H2AX)-positive cells, enhanced TIN2- γ H2AX colocalisation, impaired replication fork progression and induced telomere abnormalities without marked bulk telomere shortening. These changes were followed by cell cycle disturbance, reduced proliferation, senescence, mitochondrial damage and delayed apoptosis. The compounds also reduced migration, colony formation, spheroid growth, angiogenesis and microtumour growth. Transcriptomic analysis showed suppression of deoxyribonucleic acid (DNA) replication and cell cycle genes, with activation of stress and apoptosis pathways. These findings support that A822 derived novel TRFiN compounds act through telomere and replication stress, modulating the function of shelterin proteins, rather than through immediate, nonspecific toxicity. TRFiN49 showed the strongest profile and is a promising lead compound for telomere directed therapy.

** niepotrzebne skreślić*

*** dotyczy rozpraw doktorskich napisanych w innych językach, niż polski lub angielski*

“To all those who lost their lives to cancer, leaving behind unspoken words, unfinished dreams, and loved ones who still carry their memory with love.”

– Anoop Kallingal

Abstract

Cancer cell persistence depends on the ability to maintain protected chromosome ends during repeated cell division. Telomeres and the shelterin complex prevent chromosome ends from being recognised as DNA breaks and support telomere replication. Among shelterin proteins, telomeric repeat binding factor 1 (TRF1) and TRF1 interacting nuclear factor 2 (TIN2) are important for telomere replication and shelterin organisation. Because uncontrolled proliferation is a central feature of cancer, disturbing the TRF1-TIN2 axis may create a selective weakness by converting telomeres into sites of replication stress and DNA damage. Although telomerase and telomere length have been widely studied as anticancer targets, less is known about how small molecules that affect shelterin protein interactions produce downstream cellular responses. The main research gap addressed in this thesis was the limited mechanistic understanding of pharmacological modulation of the TRF1-TIN2 interface. The aim was to characterise novel A822 derived compounds designed to modulate telomeric protein function and to determine whether their biological activity was consistent with shelterin dysfunction, telomere associated damage, and loss of proliferation. A822 was used as the parent scaffold, and its derivatives were selected by virtual screening against the TIN2 binding region of the TRF1-TRFH domain. The compounds were examined using cytotoxicity and selectivity screening, surface plasmon resonance (SPR), molecular docking and molecular dynamics (MD), live cell imaging, flow cytometry, immunofluorescence, western blotting, DNA fibre analysis, telomere fluorescence *in situ* hybridisation, spheroid assays, wound healing assays, chorioallantoic membrane (CAM) assays, *in ovo* microtumour analysis, and transcriptomic profiling. MCF7 and HeLa cells were used as the main mechanistic cell models. The main finding of this thesis is that selected A822 derived TRFiN compounds do not act primarily through rapid bulk telomere shortening, but rather by inducing functional telomere instability linked to shelterin disturbance. TRFiN72, TRFiN49, TRFiN67b, and A822 showed low micromolar activity in cancer cell lines, and TRFiN72 showed the best balance between cytotoxicity and selectivity in breast and colon cancer models. TRFiN41, TRFiN49, and TRFiN72 were selected for detailed study. A822 and selected derivatives bound directly to TRF1, and molecular simulations supported their interaction with the TRF1 TRFH domain. In cancer cells, the active compounds increased γ H2AX positive cells, enhanced TIN2- γ H2AX colocalisation, impaired replication fork progression, altered cell cycle distribution, reduced migration, inhibited spheroid and colony growth, and induced mitochondrial dysfunction, senescence, and apoptosis. Telomere analysis showed increased telomere signal loss, doublets, and chromosome fusions, while overall telomere fluorescence changed only moderately. Transcriptomic analysis supported suppression of DNA replication and cell cycle programs together with activation of stress, senescence, and apoptosis pathways. These findings support the TRF1-TIN2 axis as a promising telomere directed anticancer target. The study contributes to the field by connecting small molecule modulation of a shelterin interface with telomere dysfunction, replication stress, and tumour suppressive cell fate responses, and identifies TRFiN49, TRFiN72, and TRFiN41 as potential lead compounds for future telomere based anticancer drug development.

Streszczenie

Utrzymanie żywotności komórek nowotworowych zależy od ich zdolności do ochrony końców chromosomów podczas kolejnych podziałów. Telomery oraz kompleks shelterinowy zabezpieczają końce chromosomów przed rozpoznaniem ich jako pęknięcia DNA i wspierają prawidłową replikację telomerów. Wśród białek shelterinowych czynnik wiążący powtórzenia telomerowe 1 (TRF1) oraz jądrowy czynnik 2 oddziałujący z TRF1 (TIN2) odgrywają istotną rolę w replikacji telomerów i organizacji całego kompleksu. Ponieważ niekontrolowana proliferacja jest jedną z głównych cech nowotworów, zaburzenie oddziaływania TRF1-TIN2 może wywołać wybiórczą wrażliwość komórek nowotworowych poprzez przekształcenie telomerów w miejsca stresu replikacyjnego i uszkodzeń DNA. Telomeraza i długość telomerów były szeroko badane jako cele terapii przeciwnowotworowej, jednak mechanizmy działania małych cząsteczek wpływających na oddziaływania pomiędzy białkami shelterinowymi pozostają słabiej poznane. Główną luką badawczą podjętą w tej rozprawie był ograniczony poziom wiedzy na temat komórkowych skutków farmakologicznego zaburzenia oddziaływania TRF1-TIN2. Celem pracy była charakterystyka nowych związków będących pochodnymi A822, zaprojektowanych jako modulatory funkcji białek telomerowych, oraz określenie, czy ich aktywność biologiczna jest zgodna z mechanizmem działania opartym na dysfunkcji shelteriny, uszkodzeniu telomerów i zahamowaniu proliferacji. A822 zastosowano jako strukturę wyjściową, a jego pochodne wybrano na podstawie wirtualnego skringu ukierunkowanego na region wiążący TIN2 w domenie TRFH białka TRF1. Związki analizowano z wykorzystaniem badań cytotoksyczności i selektywności działania, powierzchniowego rezonansu plazmonowego, dokowania molekularnego, dynamiki molekularnej, obrazowania komórek żywych, cytometrii przepływowej, immunofluorescencji, western blottingu, analizy włókien DNA, fluorescencyjnej hybrydyzacji *in situ* telomerów, testów na sferoidach, testu gojenia rany, testu na błonie kosmówkowo-omoczniowej, analizy mikroguza *in ovo* oraz profilowania transkryptomycznego. Komórki MCF7 i HeLa wykorzystano jako główne modele mechanistyczne. Główną konkluzją wynikającą z tej pracy jest to, że wybrane pochodne A822 z grupy TRFiN nie działają głównie przez szybkie globalne skracanie telomerów, lecz przez indukcję funkcjonalnej niestabilności telomerów związanej z zaburzeniem shelteriny. TRFiN72, TRFiN49, TRFiN67b i A822 wykazywały aktywność w zakresie niskich stężeń mikromolowych, a TRFiN72 charakteryzował się najlepszą równowagą między cytotoksycznością i selektywnością w modelach raka piersi i jelita grubego. Do szczegółowych badań wybrano TRFiN41, TRFiN49 i TRFiN72. A822 oraz wybrane pochodne wiązały się bezpośrednio z TRF1, a symulacje molekularne potwierdziły ich oddziaływanie z domeną TRFH. W komórkach nowotworowych aktywne związki zwiększały odsetek komórek γ H2AX-dodatnich, nasilały kolokalizację TIN2- γ H2AX, zaburzały progresję widełek replikacyjnych, zmieniały rozkład cyklu komórkowego, ograniczały migrację oraz hamowały wzrost kolonii i sferoidów. Jednocześnie indukowały dysfunkcję mitochondriów, starzenie komórkowe i apoptozę. Analiza telomerów wykazała zwiększoną utratę sygnału telomerowego, obecność dubletów oraz fuzje chromosomowe, przy jedynie umiarkowanych zmianach całkowitej fluorescencji telomerowej. Profilowanie transkryptomyczne potwierdziło zahamowanie programów replikacji DNA i cyklu komórkowego oraz aktywację szlaków stresu, starzenia i apoptozy. Wyniki te wskazują, że oś TRF1-TIN2 stanowi wartościowy i obiecujący telomerowo ukierunkowany cel przeciwnowotworowy, a TRFiN49, TRFiN72 i TRFiN41 są potencjalnie obiecującymi związkami wiodącymi dla dalszego rozwoju leków przeciwnowotworowych opartych na modulacji telomerów.

Acknowledgements

I am filled with deep gratitude as I reflect on the journey that led to the completion of this PhD thesis. This work would not have been possible without the guidance, support, encouragement, and kindness of many people who stood with me throughout this important chapter of my life.

First and foremost, I express my deepest gratitude to my supervisor, **Prof. Dr. Maciej Bagiński**, Professor, Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology. His excellent guidance, constant encouragement, valuable suggestions, constructive comments, and scientific discussions greatly shaped this research work. I am sincerely thankful for his patience, care, kindness, and continuous support, which helped me overcome many difficult stages of this PhD journey.

I extend my sincere thanks to my colleagues and fellow researchers in the Department of Pharmaceutical Technology and Biochemistry at Gdańsk University of Technology for creating a supportive and stimulating scientific environment. I am especially thankful to **Dr. Natalia Maciejewska**, **Dr. Monika Pawłowska**, **Dr. Elżbieta Zabrocka**, **Dr. Mateusz Olszewski**, **Mr. Aravind Ayyolath**, **Mr. Radosław Krzemieniecki**, **Mr. Deepak Sharma** and **Ms. Agnieszka Kurdyn** for their help, cooperation, discussions, and encouragement during my research work.

I also express my heartfelt gratitude to **Dr. Sławomir Makowiec**, Head of the Department of Organic Chemistry, and to my colleagues **Ms. Alicja Trocka** and **Ms. Anna Hromova** for their support and cooperation. I am thankful to **Dr. Wioletta Brankiewicz** from the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and Medical University of Gdańsk for her valuable assistance. I also gratefully acknowledge **Mr. Varun T. K.** from the Centre for Integrative Omics Data Science, Yenepoya (Deemed to be University), India, and **Mr. Gouthaman P. P.** from the Department of Biology, College of Science, United Arab Emirates University, UAE, for their collaboration and scientific contributions.

I am deeply thankful to **Prof. Dr. E. Jayadevi Variyar**, Department of Biotechnology and Microbiology, Kannur University, Kerala, India, for her constant motivation, care, and support from the beginning of my research journey.

I also thank all my friends, fellow researchers, teaching staff, and non-teaching staff of the Department of Pharmaceutical Technology and Biochemistry at Gdańsk University of Technology for their help, encouragement, and kindness.

Finally, I express my deepest gratitude to my beloved family members for their unconditional love, sacrifices, patience, selflessness, and unwavering support. They have stood as strong pillars in my life, guiding me from the beginning and giving me the strength to reach this stage. Above all, I thank the Almighty for the blessings, strength, and grace that made this journey possible.



This research was financially supported by the National Science Centre, Poland (NCN), under the OPUS programme, grant agreement No. UMO-2023/49/B/NZ7/03456, dated 29 January 2024. Principal Investigator: Prof. Maciej Bagiński, PhD.

Contents

Abstract	i
Streszczenie	ii
Acknowledgements	iii
List of Figures	vii
List of Tables	ix
List of Abbreviations	x
Chapter 1: Introduction	1
1.1 Cancer burden and the need for durable therapy	1
1.2 Treatment resistance, relapse, and metastatic progression	2
1.3 Replicative immortality as a common requirement in cancer	3
1.4 Telomere biology and cancer persistence	3
1.4.1 Telomere architecture and chromosome-end protection	4
1.4.2 Telomere shortening, senescence, and crisis	5
1.4.3 Telomere maintenance mechanisms in cancer	6
1.4.4 Shelterin mediated protection of chromosome ends	6
1.4.5 Telomere replication stress as a therapeutic vulnerability	7
1.5 The TRF1-TIN2 interaction as an anticancer target	8
1.6 Biological and structural rationale for targeting TRF1-TIN2	8
1.7 Previous telomere directed anticancer strategies	9
1.8 Research gap, hypothesis, and thesis rationale	9
Chapter 2: Aims and Objectives	11
2.1 Research hypothesis	11
2.2 Aim of the research	11
2.3 Research questions	11
2.4 Research objectives	12
2.5 Central research strategy	13
2.6 Significance and novelty	14
Chapter 3: Review of literature	15
3.1 Shelterin complex: composition, assembly, and core functions	15
3.1.1 Protein-protein interactions within shelterin	15
3.1.2 Coordination of end protection and telomere replication	17
3.2 Shelterin architecture and structural organisation	19
3.2.1 Stoichiometry and multivalency at telomeres	19
3.2.2 Reconstitution studies and assembly routes	20
3.2.3 Structural organisation on chromatin	21
3.3 Telomere protection and control of DNA damage responses	22
3.3.1 The chromosome end protection problem	22
3.3.2 Control of ATM, ATR, NHEJ, HDR, and end processing	24
3.4 TRF1 structure, telomere replication, and regulation	26
3.4.1 TRF1 domain organisation and interaction surfaces	26
3.4.2 TRF1 in telomere replication and replication stress	27
3.4.3 Regulation of TRF1 and its relevance to cancer therapy	28
3.5 TIN2: scaffold function, isoforms, and telomere regulation	30
3.5.1 Interaction motifs, structural organisation, and disease associated mutations	30
3.6 The TRF1-TIN2 interaction: structural basis and regulation	32
3.7 Anticancer strategies targeting shelterin and TRF1-TIN2	35
3.8 Non-canonical and extra-telomeric roles of shelterin proteins	37
3.8.1 Extra-telomeric functions of RAP1	37
3.8.2 Extra-telomeric functions of TRF2	38

Chapter 4: Materials and Methods	40
4.1 Selection and synthesis of compounds	40
4.2 Cell culturing and maintenance	40
4.3 Molecular docking and molecular dynamics simulations	41
4.3.1 Molecular docking	41
4.3.2 Molecular dynamics simulations and trajectory analysis	41
4.4 Cell cytotoxic evaluation	42
4.5 Live cell imaging, growth area analysis, cell tracking, and trypan blue assay	43
4.6 Surface plasmon resonance analysis	43
4.7 Sample preparation and acquisition for flow cytometry analysis	43
4.8 Cell cycle analysis by BrdU and propidium iodide staining	44
4.9 DNA fibre assay for analysis of replication fork progression	44
4.10 Analysis of DNA damage by γ H2AX and 7-AAD staining	45
4.11 Assessment of mitochondrial transmembrane potential by JC-1 staining	46
4.12 Transmission electron microscopy (TEM) analysis	46
4.13 Analysis of apoptosis and caspase-3/7 activation by flow cytometry	47
4.14 General immunofluorescence staining procedure	47
4.15 Cellular senescence analysis by flow cytometry and β galactosidase staining	48
4.16 Colony formation assay	48
4.17 Three dimensional spheroid formation and fluorescence imaging	49
4.18 Wound healing assay for cell migration analysis	49
4.19 CAM assay for angiogenesis and tumour growth analysis	50
4.20 Cryosectioning and haematoxylin and eosin staining of CAM tissue and embryonic organs	50
4.21 Acridine orange staining for analysis of autophagy associated acidic vesicular organelles	51
4.22 Telomere fluorescence in situ hybridisation (Telo-FISH)	51
4.22.1 Telo-FISH in metaphase spreads	52
4.22.2 Flow fluorescence in situ hybridisation (Flow-FISH)	52
4.23 Western blot analysis of protein expression	53
4.24 RNA sequencing library preparation and transcriptome data analysis	54
4.24.1 RNA isolation and quality assessment	54
4.24.2 Library preparation and sequencing	54
4.24.3 RNA-seq read processing and alignment	54
4.24.4 Differential gene expression analysis	55
4.24.5 Functional enrichment analysis	55
4.24.6 Data visualisation	55
4.25 Software, computational tools and statistical analysis	55
Chapter 5: Results	57
5.1 Selection of lead compounds for biological evaluation	57
5.2 Cytotoxicity and selectivity profile of A822 and its derivatives	59
5.3 SPR analysis of TRF1 binding and selection of lead compounds for further biological evaluation	61
5.4 γ H2AX induction and TIN2- γ H2AX colocalisation after treatment with A822 and TRFiN derivatives	63
5.5 Molecular dynamics analysis of the TRF1-TRFH domain in apo and ligand bound states	65
5.6 Effects of TRFiN compounds and 4OHT on morphology, cell growth, motility, and cell death in MCF7 and HeLa cells	70
5.7 Cell cycle redistribution and replication fork progression after treatment with TRFiN compounds	73
5.8 Time dependent induction of DNA damage in HeLa and MCF7 cells	77
5.9 Loss of mitochondrial membrane potential and mitochondrial structural damage	79
5.10 Induction of apoptosis and caspase-3/7 activation after treatment with TRFiN compounds	82
5.11 Treatment with TRFiN compounds promotes progressive senescence in MCF7 and HeLa cells	85
5.12 TRFiN compounds suppress cell migration in a dose dependent manner in MCF7 and HeLa cells	87
5.13 TRFiN compounds suppress spheroid growth in three dimensional culture and alter spheroid viability	89
5.14 TRFiN compounds reduce clonogenic growth and show cell line dependent accumulation of acidic vesicular organelles	91

5.15 TRFiN compounds inhibit chorioallantoic membrane angiogenesis and reduce in ovo microtumour growth without evident organ toxicity.....	95
5.16 TRFiN compounds increase telomere aberrations with only limited reduction in telomere fluorescence intensity.....	99
5.17 TRFiN compounds induce protein changes consistent with DNA damage response, cell cycle suppression, senescence, and apoptotic signalling.....	101
5.18 Pathway level transcriptomic profiling of TRFiN41, TRFiN49, TRFiN72 and A822 in MCF7 and HeLa cells	104
5.19 Mechanism of action of TRFiN compounds based on pathway level transcriptomic analysis.....	107
Chapter 6: Discussion	110
6.1 Targeting the TRF1-TIN2 interaction and relation among the findings.....	110
6.2 Cytotoxicity, selectivity and selection of lead compounds	110
6.3 SPR and molecular simulation	111
6.4 DNA damage, telomere abnormalities and replication stress	112
6.5 Cell cycle changes, senescence and apoptosis.....	113
6.6 Migration, long term growth and in ovo activity.....	114
6.7 Protein expression and transcriptomic changes and comparison of the lead compounds	115
6.8 Limitations and scope for further research.....	116
Chapter 7: Conclusion	118
Bibliography	120
Appendix	131
Scientific Achievements	142

List of Figures

Figure 1.1 Global cancer burden in 2022	2
Figure 1.2 Shelterin structure and function	4
Figure 1.3 T-loop formation and protection.....	5
Figure 3.1 Shelterin complex	16
Figure 3.2 Shelterin mediated repression of DNA damage signalling	18
Figure 3.3 Structure of the TIN2-TPP1-TRF2 complex	21
Figure 3.4 Shelterin mediated repression of telomeric DNA damage signalling.....	25
Figure 3.5 Structural similarity of the TIN2 TRFH domain to the TRFH domains of TRF1 and TRF2	31
Figure 5.1 Virtual screening based selection of A822 and its derivatives targeting the TRF1-TIN2 interaction site	58
Figure 5.2 Heat map analysis of IC ₅₀ values and selectivity index together with representative dose response curves for A822 and selected TRFiN derivatives	60
Figure 5.3 SPR sensorgrams of A822, TRFiN derivatives, and the TIN2 peptide binding to TRF1	62
Figure 5.4 γ H2AX induction and TIN2- γ H2AX colocalisation after treatment with A822 and selected TRFiN derivatives	64
Figure 5.5 Molecular dynamics analysis of the TRF1 TRFH domain (3BQO) in complex with A822, TRFiN41, TRFiN49 and TRFiN72 over 1000 ns	66
Figure 5.6 Principal component and free energy landscape analysis of the TRF1 TRFH domain in the apo form and in complex with A822, TRFiN41, TRFiN49 and TRFiN72 after 1000 ns molecular dynamics simulation	69
Figure 5.7 Effects of TRFiN compounds and 4OHT on morphology, cell growth, motility, and cell death in MCF7 and HeLa cells	71
Figure 5.8 Effect of TRFiN compounds and 4OHT on cell cycle distribution in HeLa and MCF7 cells at different time points	75
Figure 5.9 Effect of TRFiN compounds and 4OHT on PI/BrdU profile and replication fork progression in HeLa and MCF7 cells	76
Figure 5.10 Effect of TRFiN compounds and 4OHT on DNA damage in HeLa and MCF7 cells	78
Figure 5.11 Effect of TRFiN compounds and 4OHT on mitochondrial membrane potential in HeLa and MCF7 cells.....	80
Figure 5.12 Effect of TRFiN compounds and 4OHT on mitochondrial ultrastructure in HeLa and MCF7 cells	81
Figure 5.13 Effect of TRFiN compounds and 4OHT on apoptosis in HeLa and MCF7 cells.....	83
Figure 5.14 Effect of TRFiN compounds and 4OHT on caspase-3/7 activation and mitochondrial apoptosis-associated changes in HeLa and MCF7 cells.....	85
Figure 5.15 Effect of TRFiN compounds and 4-hydroxytamoxifen on senescence in MCF7 and HeLa cells.....	86
Figure 5.16 Effect of TRFiN compounds and sorafenib on wound healing and migration in MCF7 and HeLa cells	88
Figure 5.17 Effect of TRFiN compounds and 4-hydroxytamoxifen on spheroid growth and spheroid viability in MCF7 and HeLa cells	91
Figure 5.18 Effect of TRFiN compounds on colony formation in HeLa and MCF7 cells.....	92
Figure 5.19 Effect of TRFiN compounds and 4-hydroxytamoxifen on acidic vesicular organelle accumulation in HeLa and MCF7 cells	93
Figure 5.20 Effect of TRFiN compounds and A822 on chorioallantoic membrane angiogenesis	95
Figure 5.21 Effect of TRFiN compounds and A822 on in ovo microtumour growth	97
Figure 5.22 Histological evaluation of chicken embryo organs after treatment with TRFiN compounds.....	98
Figure 5.23 Effect of TRFiN compounds and A822 on telomere integrity and TIN2/TRF1/2 colocalisation in HeLa and MCF7 cells	100
Figure 5.24 Effect of TRFiN compounds and A822 on protein expression in MCF7 and HeLa cells.....	103

Figure 5.25 Differential gene expression profiles after 168 h treatment with TRFiN compounds and A822 in HeLa and MCF7 cells.....	104
Figure 5.26 Chromosomal distribution of pathway-associated genes and top 10 KEGG pathway enrichment after 168 h treatment with TRFiN compounds and A822.....	106
Figure 5.27 The schematic diagram of proposed common mechanism of action of TRFiN compounds.....	108
Appendix figures	
Figure 1 Raw data of western blot 1 (full blots).....	138
Figure 2 Raw data of western blot 2 (full blots).....	139
Figure 3 Raw data of western blot 3 (full blots).....	140
Figure 4 Distribution of differentially expressed genes across treatment groups	141
Figure 5 Distribution of mRNA expression levels of shelterin complex across treatment groups	141

List of Tables

Table 5.1 SPR binding parameters of A822, TRFiN derivatives, and TIN2 peptide against TRF1 63

Table 5.2 MM/GBSA binding energy summary for A822 and selected derivatives 68

Appendix tables

Table 1 Cell lines used in the study, tissue origin, and routine culture conditions 133

Table 2 IC₅₀ concentration of compounds and standard drugs 134

Table 3 List of antibodies and fluorophores used 135

Table 4 Structure of novel A822 derivatives (TRFiN series) 137

List of Abbreviations

4OHT	<i>4-hydroxytamoxifen</i>
6-thio-dG	<i>6-thio-2'-deoxyguanosine</i>
7-AAD	<i>7-aminoactinomycin D</i>
A822	<i>Parent small-molecule compound used as the reference scaffold</i>
AIF	<i>Apoptosis-inducing factor</i>
ALT	<i>Alternative lengthening of telomeres</i>
ANOVA	<i>Analysis of variance</i>
AO	<i>Acridine orange</i>
APB	<i>ALT-associated promyelocytic leukaemia body</i>
ASR	<i>Age-standardised rate</i>
ATM	<i>Ataxia telangiectasia mutated kinase.</i>
ATR	<i>Ataxia telangiectasia and Rad3-related kinase</i>
AVO	<i>Acidic vesicular organelle</i>
BAX	<i>BCL2-associated X protein</i>
BCL2	<i>B-cell lymphoma 2</i>
BIR	<i>Break-induced replication</i>
BLM	<i>Bloom syndrome helicase</i>
BrdU	<i>Bromodeoxyuridine</i>
BSA	<i>Bovine serum albumin</i>
CAM	<i>Chick chorioallantoic membrane</i>
CDKN1A	<i>Cyclin-dependent kinase inhibitor 1A, p21</i>
CO ₂	<i>Carbon dioxide</i>
CPM	<i>Counts per million</i>
DAPI	<i>4',6-diamidino-2-phenylindole</i>
DCCM	<i>Dynamic cross-correlation matrix</i>
DDR	<i>DNA damage response</i>
DEG	<i>Differentially expressed gene</i>
DMSO	<i>Dimethyl sulfoxide</i>
DNA	<i>Deoxyribonucleic acid</i>
DNA-PK	<i>DNA-dependent protein kinase</i>
DSB	<i>Double-strand break</i>
EDTA	<i>Ethylenediaminetetraacetic acid</i>
ETP	<i>Etoposide</i>
FBS	<i>Fetal bovine serum</i>
FCCP	<i>Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine</i>
FEL	<i>Free energy landscape</i>
FISH	<i>Fluorescence in situ hybridisation</i>
FITC	<i>Fluorescein isothiocyanate</i>
Flow-FISH	<i>Flow fluorescence in situ hybridisation</i>
G0/G1	<i>Gap 0/gap 1 phase of the cell cycle</i>
G2/M	<i>Gap 2/mitosis phase of the cell cycle</i>
GLOBOCAN	<i>Global Cancer Observatory cancer statistics database</i>
GO	<i>Gene Ontology</i>
H2AX	<i>Histone H2AX</i>
γH2AX	<i>Phosphorylated histone H2AX</i>
HDR	<i>Homology-directed repair</i>
HTVS	<i>High-throughput virtual screening</i>
IC ₅₀	<i>Half-maximal inhibitory concentration</i>
IF	<i>Immunofluorescence</i>

JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
k_a	Association rate constant
k_d	Dissociation rate constant
KD	Equilibrium dissociation constant
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAC	Latin America and the Caribbean
MD	Molecular dynamics
MM/GBSA	Molecular mechanics/generalized Born surface area
MRN	MRE11-RAD50-NBS1 complex
MTS	Multitelomeric signals
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHEJ	Non-homologous end joining
NGS	Next-generation sequencing
OB	Oligosaccharide/oligonucleotide-binding fold
OCT	Optimal cutting temperature compound
OPLS	Optimised potentials for liquid simulations
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PBS-EDTA	Phosphate-buffered saline-ethylenediaminetetraacetic acid
PBST	Phosphate-buffered saline with Tween 20
PCA	Principal component analysis
PCC	Pearson correlation coefficient
PDB	Protein Data Bank
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K/AKT	Phosphoinositide 3-kinase/protein kinase B pathway
PML	Promyelocytic leukaemia protein
PNA	Peptide nucleic acid
POT1	Protection of telomeres 1
PPI	Protein-protein interaction
PVDF	Polyvinylidene difluoride
RAP1	Repressor/activator protein 1
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
ROS	Reactive oxygen species
RSEM	RNA-Seq by Expectation-Maximization
RT	Room temperature
RU	Response units
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	Selectivity index
SPR	Surface plasmon resonance
TBM	TRFH-binding motif
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
Telo-FISH	Telomere fluorescence in situ hybridisation
TEM	Transmission electron microscopy
TERC	Telomerase RNA component
TERF1	Gene encoding TRF1
TERF2	Gene encoding TRF2

TERF2IP	<i>Gene encoding RAP1</i>
TERT	<i>Telomerase reverse transcriptase</i>
TIF	<i>Telomere dysfunction-induced focus</i>
TIN2	<i>TRF1-interacting nuclear factor 2</i>
TINF2	<i>Gene encoding TIN2</i>
TMM	<i>Telomere maintenance mechanism</i>
TOMM20	<i>Translocase of outer mitochondrial membrane 20</i>
TPP1	<i>TIN2-interacting protein 1</i>
TRF1	<i>Telomeric repeat-binding factor 1</i>
TRF2	<i>Telomeric repeat-binding factor 2</i>
TRFH	<i>TRF homology domain</i>
TRFiN	<i>TRF1-TIN2 interaction modulator compound series</i>
VEGF-A	<i>Vascular endothelial growth factor A</i>
WB	<i>Western blot</i>
XP	<i>Extra precision docking mode</i>

Chapter 1

Introduction

Cancer remains a major global health problem because of its high incidence, mortality, and frequent recurrence after treatment. The Global Cancer Observatory estimated approximately 20 million new cases and 9.7 million cancer related deaths worldwide in 2022¹. The number of people living with or after cancer treatment is also increasing, creating a need for therapies that provide durable disease control¹. A defining requirement of malignant growth is replicative immortality, which allows tumour cells to continue dividing despite normal barriers to proliferation². Telomeres and their associated proteins lie at the centre of this process because they protect chromosome ends from being recognised and repaired as DNA breaks³. This Introduction links cancer persistence to telomere maintenance, introduces shelterin and the telomeric repeat binding factor 1 (TRF1)-TRF1-interacting nuclear factor 2 (TIN2) interaction, and defines the research gap.

1.1 Cancer burden and the need for durable therapy

Cancer incidence and mortality vary between regions because of differences in population age, exposure to risk factors, access to screening, and the availability of treatment¹. Nevertheless, the 2022 global estimates show that the overall burden remains high¹. The growing number of cancer cases associated with population growth and ageing will create increasing pressure on healthcare systems^{1,4}. Major improvements have been achieved through surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. However, many patients still develop recurrent or progressive disease because tumour cell populations are heterogeneous and can adapt during treatment. Resistance may be present before treatment or may develop through the selection of resistant cells during therapy^{5,6}. These observations support the search for vulnerabilities that are repeatedly required for tumour growth and cannot be readily bypassed. Global cancer incidence and mortality are summarised in Figure 1.1.

The relationship between incidence and mortality also shows why cancer cannot be considered a single clinical problem. Tumours arising in different tissues vary in their rate of progression, sensitivity to treatment, and probability of early detection. Even within one tumour type, molecular subgroups can respond differently to the same treatment. A therapy may therefore produce a strong initial reduction in tumour burden without eliminating the cells capable of rebuilding the disease. Long term control depends on removing or permanently suppressing these surviving populations. This distinction between initial response and durable control is important for the present thesis because telomere maintenance is not required only at the beginning of tumour development. It is required repeatedly during continued growth, recovery after treatment, and formation of new lesions. Improved survival has also created a need to consider delayed recurrence and the long term effects of treatment. Residual malignant cells may remain clinically undetectable for months or years before resuming proliferation. During this period, they can be exposed to immune pressure, limited nutrients, altered tissue conditions, and additional treatment. A biological process that supports survival only during rapid growth may not be sufficient to explain such persistence. By contrast, maintenance of chromosome ends remains necessary whenever a surviving cell re-enters the cell cycle. Telomere protection can therefore be viewed as a continuous requirement that links early tumour expansion with later recurrence.

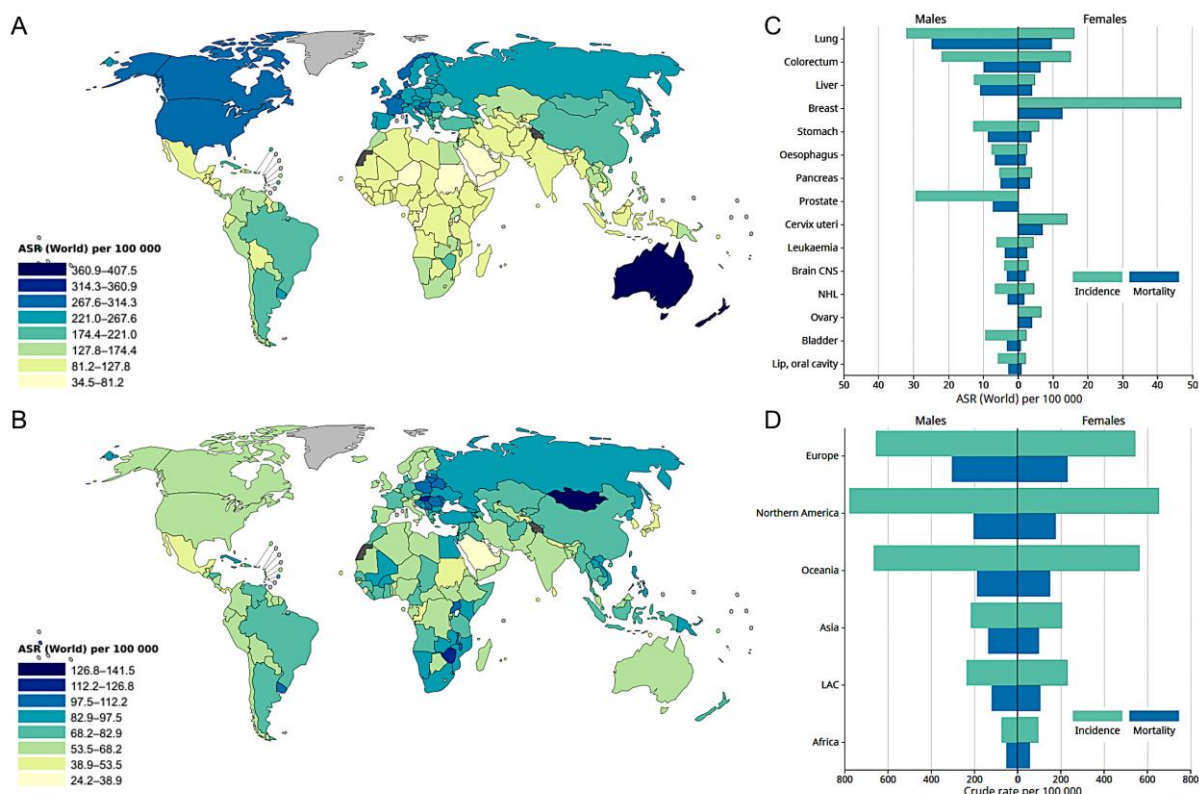


Figure 1.1. Global cancer burden in 2022: (A) world map of age standardised incidence rate (ASR, World) for all cancers, both sexes, ages 0-74; (B) world map of age standardised mortality rate (ASR, World) for all cancers, both sexes, ages 0-74; (C) age standardised incidence and mortality rates (ASR, World) for males and females, showing the top cancer sites; and (D) continental crude incidence and mortality rates per 100,000 for all cancers in males and females¹.

1.2 Treatment resistance, relapse, and metastatic progression

Treatment failure is commonly associated with intrinsic or acquired resistance, recurrence, and metastatic spread^{6,7}. Resistant cells may carry pre-existing genetic changes, acquire new mutations, alter drug transport or metabolism, activate alternative signalling pathways, or enter reversible cell states that reduce their sensitivity to treatment^{6,7}. The tumour microenvironment can further protect malignant cells by modifying oxygen and nutrient availability, extracellular matrix interactions, inflammatory signalling, and immune activity⁸. Cells that survive treatment can later re-enter the cell cycle and repopulate the primary site or establish metastatic lesions^{7,9}. Metastasis is especially important because successful dissemination requires tumour cells to survive several restrictive steps, including local invasion, circulation, colonisation, and renewed growth in a distant tissue⁹. Although these processes involve different mechanisms, all surviving clones must retain the ability to proliferate. Long term chromosome stability and telomere protection are therefore relevant to primary tumour growth, recurrence, and metastasis.

Tumour heterogeneity operates at several levels. Genetic heterogeneity arises from mutations, copy number changes, chromosome rearrangements, and epigenetic differences between cells. Non-genetic heterogeneity arises when cells reversibly change their metabolism, differentiation state, stress response, or interaction with surrounding tissue. These differences can allow a small subpopulation to tolerate treatment even when most tumour cells are sensitive. Once treatment removes the sensitive population, surviving cells face less competition and can expand. The resulting recurrent tumour may therefore differ from the tumour present at diagnosis. A target intended for durable treatment should remain important across these changing cell states^{10,11}. Metastatic progression imposes additional stress. Disseminating cells must detach from the primary tumour, migrate through tissue, enter the circulation, survive there, leave the vasculature, and adapt to a new

organ. Most cells do not complete this sequence. Those that do must establish a new proliferating population from a very small number of surviving cells. This requires repeated DNA replication under conditions that may be unfavourable. Chromosomal instability during this period can either prevent successful colonisation or, if insufficiently controlled, increase genome variation. Telomere biology can therefore have two opposing effects: telomere dysfunction can promote genome instability during tumour evolution, but excessive or persistent dysfunction can stop proliferation. Therapeutic intervention aims to shift this balance beyond the level that cancer cells can tolerate^{12,13}.

1.3 Replicative immortality as a common requirement in cancer

A clinically detectable tumour develops through many rounds of cell division and must continue dividing during recurrence and metastatic growth. This sustained proliferative capacity is described as replicative immortality². Normal somatic cells have a limited proliferative lifespan because telomeres shorten with each round of DNA replication and eventually trigger checkpoint responses. Senescence prevents further division, whereas a crisis can cause extensive chromosome instability and cell death. Cancer cells must avoid or overcome these barriers². Most tumours achieve this by maintaining telomeres through telomerase reactivation, while a smaller group uses alternative lengthening of telomeres¹⁴. Telomere maintenance does not act alone. Chromosome ends must also remain protected during each cell cycle. Tumour cells therefore depend on both the preservation of telomeric DNA and the continued function of the proteins that organise and protect it.

Replicative immortality does not mean that cancer cells divide without stress or without limits. Instead, it means that the population has acquired mechanisms that prevent telomere dependent barriers from permanently stopping growth. Individual cells may still undergo senescence, apoptosis, mitotic failure, or immune clearance¹⁵. Tumour progression continues because enough cells maintain telomeres and preserve chromosome end function. This population level view is important when interpreting drug responses. A moderate reduction in short term viability may be less informative than a marked loss of clonogenic capacity, because colony formation measures whether treated cells retain the ability to generate a new population after the drug is removed¹⁶. Telomere maintenance is also connected to checkpoint status. Cells with functional DNA damage checkpoints can respond to a limited number of dysfunctional telomeres by entering a stable arrest. Cells with defective checkpoint pathways may continue through the cell cycle and accumulate chromosome fusions and rearrangements. This may initially increase tumour variation, but continued damage can eventually become incompatible with survival. The response to telomere directed treatment is therefore expected to depend on the cell's molecular background. The use of more than one cancer cell line in this thesis was intended to distinguish effects that were common to telomere disturbance from effects that depended on a particular checkpoint or cell death pathway^{17,18}.

1.4 Telomere biology and cancer persistence

Telomeres are specialised nucleoprotein structures located at the ends of linear chromosomes. They form a boundary between the natural chromosome terminus and the cellular systems that recognise accidental DNA breaks. An exposed chromosome end can recruit DNA damage sensors, nucleases, and ligases, leading to checkpoint activation, sequence loss, or end to end fusion. Telomeres prevent these events while still allowing replication and regulated end processing. This balance is important in cancer cells because rapid proliferation increases the frequency of replication associated intermediates that must be processed without activating a persistent DNA damage response, as summarised in Figure 1.2. Human telomeres contain non-coding repetitive DNA and a specialised protein complex called shelterin. Together, these elements suppress inappropriate DNA damage signalling and repair at chromosome ends^{19,20}. Loss of protection can produce telomere dysfunction induced foci, chromosome fusions, genome instability, senescence, or cell death. Cancer cells must maintain this protective state while continuing to replicate, which makes telomere maintenance and the shelterin complex potential therapeutic targets.

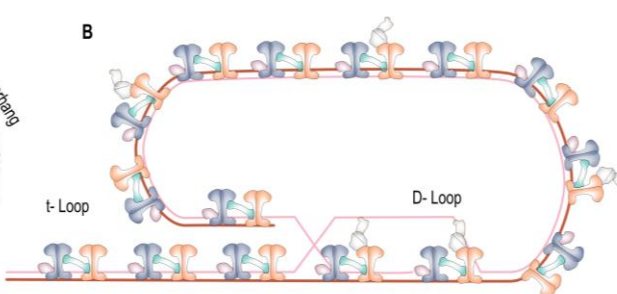


Figure 1.2. Schematic representation of human shelterin structure and function: (A) The six shelterin components are shown at double stranded and single stranded telomeric DNA. TRF1 and TRF2 proteins bind double stranded repeats as homodimers. (B) Shelterin complexes are distributed along telomeric DNA and connect duplex repeat binding with protection of the single stranded overhang²¹.

1.4.1 Telomere architecture and chromosome end protection

Human telomeric DNA sequence consists mainly of tandem TTAGGG repeats. These repeats are connected to chromosome internal coding regions through subtelomeric DNA, which contains non-coding and repeated sequences that vary between chromosome ends. The telomeric G-rich DNA strand extends beyond the complementary strand to form a 3' single stranded overhang. This DNA overhang can fold back and invade the duplex telomeric tract, producing a telomeric loop (t-loop) and a displacement loop (D-loop) at the invasion site²². The t-loop can reduce access of DNA repair proteins to the chromosome terminus, but it must be opened and re-formed during replication and end processing. Telomere protection is therefore dynamic rather than a permanently closed structural state. The duplex telomeric tract and the single stranded DNA overhang provide different binding substrates. Proteins that recognise DNA double stranded repeats organise the longer telomeric array, while proteins that recognise the overhang regulate access to the extreme chromosome end. Subtelomeric regions add another level of organisation because their sequence and chromatin state can influence transcription, replication timing, and the transition from chromosome internal DNA to terminal repeats. Telomeres should therefore be considered as structured chromatin domains rather than isolated repeated sequences. Their function depends on DNA length, local structure, protein occupancy, and changes that occur during the cell cycle.

T-loop formation provides a useful model of chromosome end concealment, but it does not explain all states of a telomere. The overhang must become accessible for processing and telomerase action, and replication forks must pass through the duplex repeat tract before a mature DNA end can be restored. Temporary opening is therefore normal. The biological problem is not simply whether the telomere is open or closed, but whether access to DNA is properly timed and whether the correct proteins are present during the transition. A compound that alters shelterin assembly may disturb this timing even if it does not completely remove shelterin from telomeric DNA. These processes are summarised in Figure 1.3. An unprotected telomere resembles a DNA double strand break and can activate DNA damage response pathways. Shelterin proteins prevent this response by limiting the activity of ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) kinases, while restricting non homologous end joining, alternative end joining, as well as homologous recombination at chromosome ends^{19,20,23}. Telomeric repeats also create replication barriers because guanine rich DNA can form G-quadruplex structures and other secondary structures that slow replication forks. Consequently, telomeres must alternate between protected and accessible states so that DNA replication, overhang processing, and telomerase action can occur without uncontrolled repair^{20,24}. This balance is particularly important in tumour cells with high replication stress.

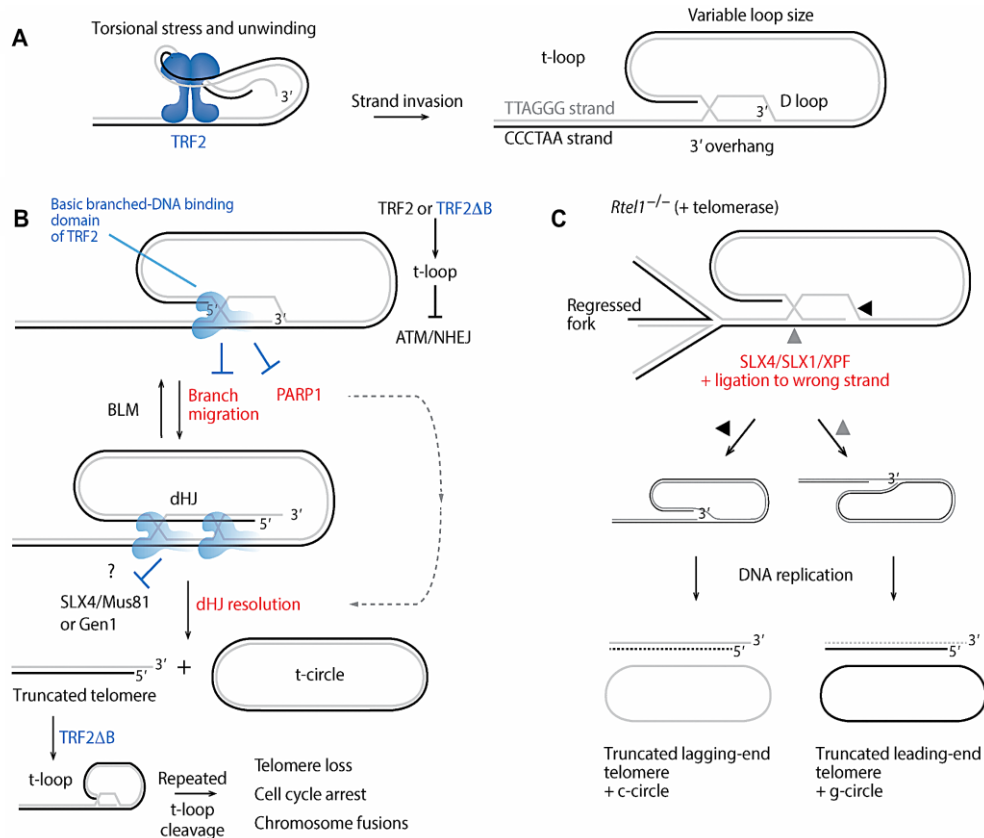


Figure 1.3. T-loop formation and protection: (A) TRF2 wraps double stranded telomeric DNA, creating topological stress that promotes 3'-overhang strand invasion and t-loop formation. (B) The TRF2 basic domain binds branched DNA at the loop base, shielding the 5' end from PARP1 and limiting branch migration and double Holliday junction (dHJ) formation; without it, HJ processing leads to telomere truncation and telomeric circles. Bloom syndrome helicase (BLM) can dissolve dHJs. (C) Rtel1 prevents replication fork arrest at t-loops; loss of Rtel1 causes fork regression and nuclease driven truncation/circle formation via a pathway distinct from (B)²³.

1.4.2 Telomere shortening, senescence, and crisis

The end replication problem arises because DNA polymerases require a primer and can only synthesise DNA in the 5'→3' direction. After removal of the final ribonucleic acid (RNA) primer from the lagging strand, the extreme chromosome end cannot be copied completely. Telomeric DNA is therefore lost during successive cell divisions^{25,26}. When telomeres become critically short or lose protection, chromosome ends activate a persistent DNA damage response that limits proliferation. In cells with intact checkpoints, this usually produces replicative senescence. If checkpoint control is defective, cells can continue dividing until they enter telomere crisis, which is characterised by chromosome fusion, breakage fusion bridge cycles, genome rearrangement, and extensive cell death²⁷. Rare cells that acquire a telomere maintenance mechanism can escape crisis and continue proliferating. Telomere shortening therefore acts as a tumour suppressive barrier, while escape from this barrier supports malignant progression.

Senescence and crisis represent different outcomes of telomere dysfunction. Senescence is generally a controlled response in which cells remain metabolically active but no longer divide. It is accompanied by long term changes in gene expression, chromatin organisation, cell morphology, and secretion. In a tumour, senescence can restrict growth, although senescent cells may also influence neighbouring cells through secreted factors. A crisis occurs when cells with inadequate checkpoints continue to divide despite severe telomere dysfunction^{28,29}. Chromosome ends fuse and are pulled apart during mitosis, creating repeated cycles of breakage and rearrangement. Most cells die during this process, but rare survivors can emerge with extensive genomic changes and an active telomere

maintenance mechanism. This distinction affects therapeutic interpretation. A telomere directed compound may not produce immediate apoptosis in every cell. Instead, damage may accumulate over several divisions until cells enter senescence or lose reproductive capacity. Time dependent experiments are therefore needed. Measurements made only after 24 or 48 hours can miss delayed phenotypes that become clear after several days. For this reason, the present study examined early DNA damage and replication responses, as well as later senescence, apoptosis, spheroid growth, and clonogenic survival.

1.4.3 *Telomere maintenance mechanisms in cancer*

Two main telomere maintenance mechanisms are found in human cancers: telomerase reactivation and alternative lengthening of telomeres (ALT). Telomerase is a ribonucleoprotein reverse transcriptase that uses its internal RNA template to add telomeric repeats. Reactivation commonly involves increased expression or altered regulation of telomerase reverse transcriptase, allowing tumour cells to maintain telomeres above the threshold that triggers persistent DNA damage signalling^{30,31}. Direct telomerase inhibition was investigated for many years before an anticancer medicine from this class was approved. Imetelstat is a first in class oligonucleotide telomerase inhibitor approved for selected adults with lower risk myelodysplastic syndromes³². It has also received marketing authorisation in the European Union³³. However, experimental studies in acute myeloid leukaemia showed that Imetelstat can alter fatty acid metabolism, increase lipid reactive oxygen species, and promote ferroptosis, which apparently may be the major mechanism of action³⁴. In solid tumours, however, telomerase inhibition may require a long treatment period before telomeres become critically short. This delay supports the development of approaches that induce telomere dysfunction more directly. ALT is a recombination dependent mechanism that maintains telomeres in the absence of telomerase³⁵. ALT positive tumours often show heterogeneous telomere lengths, ALT associated promyelocytic leukaemia bodies, and increased recombination at telomeric DNA. Current models involve break induced DNA synthesis and the use of telomeric sequences as templates for extension³⁶. Because ALT relies on DNA repair and replication pathways, changes in shelterin function can affect the access of recombination factors to telomeres. Telomerase positive and ALT positive tumours therefore use different mechanisms of repeat maintenance, but both still require sufficient chromosome end protection to avoid lethal damage.

Telomerase and ALT address repeat loss, but neither mechanism eliminates the need for shelterin. Newly synthesised telomeric DNA must still be organised, replicated in the next cell cycle, and protected from DNA repair. This provides a rationale for investigating shelterin directed treatment even when the telomere maintenance mechanism is not fully understood. A compound that interferes with end protection may act before a measurable reduction in average telomere length occurs. It may also affect cells that could otherwise compensate for telomerase inhibition by altering their telomere maintenance programme³⁷. The clinical success of Imetelstat confirms that telomerase is a druggable enzyme, but it does not mean that all telomerase positive cancers will respond similarly. Response depends on disease biology, treatment duration, baseline telomere state, and cellular pathways activated after telomerase inhibition. The ferroptosis findings in acute myeloid leukaemia further show that a telomerase inhibitor can produce metabolic and cell death effects that are not explained solely by gradual telomere shortening. Telomere directed compounds should therefore be studied with broad mechanistic assays while retaining telomere specific measurements that test the proposed primary action³⁸.

1.4.4 *Shelterin mediated protection of chromosome ends*

Shelterin contains six principal components: TRF1, telomeric repeat binding factor 2 (TRF2), repressor/activator protein 1 homologue (RAP1; TERF2IP), TIN2, adrenocortical dysplasia protein homologue commonly termed TPP1, and protection of telomeres 1 (POT1)^{19,23}. TRF1 and TRF2 bind

double stranded DNA telomeric repeats through carboxy terminal Myb/SANT (carboxy terminal myeloblastosis /Swi3, Ada2, N CoR, and TFIIB) domains and form homodimers through their TRF homology domains³⁹. POT1 binds the single stranded G-rich DNA overhang, while TPP1 recruits and stabilises POT1 and contributes to telomerase recruitment. RAP1 associates mainly with TRF2. TIN2 connects the double stranded DNA binding components to the TPP1-POT1 module and acts as the central scaffold of the complex^{19,30}. Structural studies show that TIN2 contains separate interaction surfaces for its partners, allowing shelterin assembly to be regulated through defined protein protein contacts⁴⁰. Shelterin does more than bind telomeric DNA. It suppresses specific DNA damage response and repair pathways at chromosome ends^{19,20,23}. TRF2 is mainly associated with repression of ATM signalling and classical non-homologous end joining, whereas POT1 limits ATR activation by preventing replication protein A (RPA) from binding the single stranded overhang⁴¹. TRF1 supports efficient replication of the duplex telomeric tract, while TIN2 maintains connections between shelterin modules. Removal of several shelterin components exposes chromosome ends to ATM and ATR signalling, end joining, resection, and recombination⁴². Pharmacological interference is unlikely to reproduce complete genetic deletion. A small molecule may weaken one interaction, alter the distribution of shelterin subcomplexes, or reduce recruitment of an accessory factor. Nevertheless, partial disruption can be biologically important if it produces persistent telomeric damage in rapidly dividing tumour cells.

Each shelterin component contributes to a distinct yet related part of telomere function. TRF1 supports replication of the duplex repeats and recruits proteins that help resolve replication barriers. TRF2 contributes to t-loop formation, suppresses ATM signalling, and restricts end joining. RAP1 associates with TRF2, stabilises it, and contributes to the control of recombination. TIN2 connects the main shelterin modules and influences the stability and organisation of the complex. TPP1 links TIN2 with POT1 and contributes to telomerase recruitment, while POT1 protects single stranded telomeric DNA and limits ATR activation. Because these functions are distributed across the complex, disruption of different interactions can produce different combinations of replication stress, DNA damage signalling, and repair⁴³. The modular organisation of shelterin also creates a challenge for studying the mechanism of action. A reduced abundance of one component can indirectly alter the localisation of another. A compound that binds TRF1 may alter TIN2 recruitment, POT1 positioning, or the access of replication proteins without directly binding those factors. Conversely, a general DNA damaging compound can produce telomere associated signals because telomeres are difficult genomic regions. Evidence for shelterin modulation must therefore come from several observations that agree with one another rather than from a single marker⁴⁴.

1.4.5 Telomere replication stress as a therapeutic vulnerability

Telomeric DNA is difficult to replicate due to its repetitive sequences, secondary structures, and specialised chromatin. Shelterin supports replication by controlling fork progression, telomerase access, and the CTC1-STN1-TEN1 (CST)-DNA polymerase α /primase fill in reaction at chromosome ends²³. These functions are relevant in both telomerase positive and ALT positive cells because replication failure can activate a telomeric DNA damage response even when telomeres are not critically short³⁶. Several compounds show that telomeres can be considered into key therapeutic targets. The G-quadruplex ligand RHPS4 induces telomere associated damage⁴⁵, while telomestatin promotes telomere uncapping and alters the binding of POT1 and TRF2⁴⁶. The modified nucleoside 6-thio-2'-deoxyguanosine (6-thio-dG) is incorporated into telomeres by telomerase and induces telomere dysfunction in telomerase positive cancer cells^{47,48}. These approaches act more rapidly than strategies that depend only on progressive telomere shortening and support the direct targeting of telomere protection and replication.

Average telomere length alone cannot fully describe telomere function. A cell can contain a mixture of long and short telomeres, and one critically short or unprotected chromosome end may activate a damage response even when the average fluorescence signal remains relatively high. Telomere dysfunction can also arise from replication failure, loss of shelterin occupancy, or defective overhang processing without an immediate change in total telomeric DNA. For this reason, the present study did not treat telomere shortening as the only expected outcome. Telomere associated damage foci, metaphase abnormalities, replication fork behaviour, and changes in telomere fluorescence were considered together. This distinction is important for compounds intended to disrupt protection, because their first effect may be qualitative damage at selected chromosome ends rather than a uniform reduction in telomere length.

1.5 The TRF1-TIN2 interaction as an anticancer target

The repeated requirement for telomere replication and end protection makes shelterin a possible anticancer target. Its assembly depends on a limited number of defined protein protein interactions, including TRF1-TIN2, TRF2-RAP1, TIN2-TPP1, and TPP1-POT1. These contacts connect DNA binding modules with proteins that regulate overhang protection, replication, and telomerase recruitment. Disruption of a selected interface may therefore disturb a specific function without requiring complete removal of the complex. This thesis focuses mainly on TRF1 and the TRF1-TIN2 interaction because of their roles in duplex telomere replication and shelterin organisation. The TRF1-TIN2, TRF2-RAP1, TIN2-TPP1, and TPP1-POT1 interactions form the main connections within shelterin^{19,23}. TRF1 recognises a defined TIN2 motif through a conserved surface in its TRF homology domain⁴⁹. TIN2 also contains a TRF homology like fold that binds TPP1 and TRF2 derived motifs⁴⁰. Reconstitution studies show that shelterin does not exist only as one rigid six subunit particle. Complexes with different stoichiometries and partner combinations can form⁵⁰, and the presence of TPP1 can alter how TIN2 engages TRF1 and TRF2⁵¹. Structural studies further show that shelterin assemblies are flexible and heterogeneous⁵², while live cell studies support the presence of subcomplexes with different residence times at telomeres⁵³. These findings indicate that a compound targeting one interface may alter the balance between functional shelterin states.

1.6 Biological and structural rationale for targeting TRF1-TIN2

TRF1 supports the replication of double stranded telomeric repeats, while the broader shelterin complex limits ATM and ATR dependent signalling and inappropriate DNA repair^{20,23}. TRF2 mainly suppresses ATM signalling and end joining⁴¹, and complete shelterin loss activates several damage and repair pathways simultaneously⁴². Shelterin also coordinates telomerase and CST DNA polymerase α /primase during completion of chromosome end replication²³. TRF1 is particularly important under replication stress because it reduces telomere fragility²³. The TRF1-TIN2 interface is suitable for chemical targeting because the TRF1-TRFH domain binds a short, defined TIN2 motif within a structurally characterised pocket⁴⁹. In tumour models, TRF1 inhibition induced telomeric DNA damage and reduced the growth of p53 deficient KRAS (Kirsten rat sarcoma viral oncogene homologue)-driven lung tumours⁵⁴. Whole body loss of TRF1 in adult mice caused only limited effects in normal tissues under the experimental conditions used, indicating that TRF1 inhibition may be tolerated to some extent in normal tissues⁵⁵. Some recent fragment based screening has now identified small molecules that bind the TRF1 domain and disrupt TIN2 binding⁵⁶. These findings provide further structural, biological, and chemical support for investigating TRF1-TIN2 modulation, which has been studied in our group for last several years.

TRF1-TIN2 was prioritised because it combines a defined binding surface with a function that is relevant to rapidly proliferating cells. TRF1 is positioned throughout the duplex telomeric tract and contributes to the passage and recovery of replication forks. TIN2 stabilises the connection between

TRF1 bound DNA and the remaining shelterin modules. Weakening this interaction could therefore reduce the ability of telomeres to complete replication while also altering the organisation of the protective complex. The expected result is not necessarily immediate removal of all TRF1⁵⁷. A partial reduction in stable occupancy or partner recruitment may be sufficient to increase the number of incompletely replicated or improperly protected telomeres during successive cell cycles. Protein-protein interactions were once considered difficult targets for small molecules because their surfaces are often larger and flatter than those of enzyme active sites. However, many interactions depend on a smaller set of residues that account for most of the binding energy. The TRF1 TRF homology domain recognises a short TIN2 motif within a defined region, making it more suitable for ligand discovery than a diffuse contact between two large surfaces⁴⁹. Structural information can guide docking and molecular dynamics analyses, while biochemical binding assays can test whether compounds bind TRF1. Cellular experiments are then required to determine whether this interaction produces the expected telomere-related consequences.

1.7 Previous telomere directed anticancer strategies

Earlier telomere directed anticancer strategies mainly involved telomerase inhibition or direct induction of telomere dysfunction. Imetelstat demonstrated that telomerase can be pharmacologically inhibited and provides clinical benefit in selected haematological diseases³². Its development also showed that response and tolerability depend on disease type, treatment schedule, and patient selection^{32,58}. A limitation of telomerase inhibition in many solid tumours is the time required for telomere shortening to progress. More rapid effects can be produced by damaging telomeric DNA or disturbing telomere associated proteins. RHPS4 induces telomeric DNA damage by stabilising G-quadruplex structures⁴⁵, and telomestatin can reduce POT1 and TRF2 association with telomeres⁴⁶. However, G quadruplexes also form outside telomeres, which can reduce target specificity^{45,46}.

A different strategy uses telomerase to incorporate 6-thio-dG into telomeres, thereby inducing telomere dysfunction⁴⁸. This approach has shown antitumour activity in experimental models⁴⁷, but it depends on telomerase and may not apply to ALT positive tumours. Direct targeting of shelterin may avoid this limitation. Increased expression of TRF1, TRF2, and TIN2 has been reported in some cancers⁵⁹, and genetic or pharmacological inhibition of TRF1 can reduce tumour growth⁵⁴. The recent identification of TRF1-TIN2 inhibitors provides a direct route from the interaction structure to small molecule testing⁵⁶. These studies support telomere protection as a therapeutic target while also showing the need for careful confirmation of cellular target engagement. Direct disturbance of telomere protection may offer a shorter route to growth arrest than gradual inhibition of repeat addition, but it also requires careful assessment of selectivity. Normal proliferating tissues, including haematopoietic and epithelial compartments, also require functional telomeres. A useful therapeutic window may arise if tumour cells exhibit greater replication stress, weaker checkpoints, greater dependence on specific shelterin states, or reduced capacity to recover from telomeric damage. This possibility cannot be assumed from cytotoxicity in cancer cells alone. It must be examined by comparing concentrations, exposure duration, model systems, and evidence of toxicity outside the tumour. The *in ovo* embryonic organ analysis and assessment of treatment effects on normal cellular models were therefore relevant to the broader evaluation of the compound series.

1.8 Research gap, hypothesis, and thesis rationale

The literature indicates that telomere maintenance and the shelterin complex are required for prolonged tumour growth. They also show that TRF1 is involved in telomere replication and that the TRF1-TIN2 interface can bind small molecules. However, the cellular consequences of partial pharmacological disruption remain insufficiently defined. Genetic deletion removes an entire protein, whereas a compound may alter only one interaction or change protein localisation. The resulting phenotype may therefore differ in strength, timing, and pathway dependence. This thesis addresses

that difference by examining compounds designed to disrupt shelterin function and by linking their binding properties to telomere specific and cellular responses.

Most mechanistic insights into shelterin dysfunction have been obtained from genetic depletion, conditional deletion, or engineered mutants^{23,54}. These approaches define the consequences of severe loss of function but do not fully predict the effect of a small molecule that weakens a selected interaction. Furthermore, reduced proliferation, DNA damage, apoptosis, and senescence can arise from many forms of cellular stress. A central research gap is therefore the lack of an integrated method for distinguishing shelterin related telomere dysfunction from general cytotoxicity. This requires analysis of compound binding, telomeric DNA damage, replication behaviour, telomere structure, cell cycle responses, long term cell fate, and pathway level changes in the same experimental framework.

The TRF1-TIN2 is now a reasonable protein-protein interaction (PPI) target because the interface is discrete and structurally visible^{49,56}. Early inhibitors provide biochemical evidence that the interface can be targeted by small molecules⁵⁶, but their cellular effects and downstream pathway signatures remain underexplored. For translation, it is not enough to show that a compound disrupts a peptide probe *in vitro*. It is necessary to determine whether TRF1-TIN2 disruption in cells consistently activates the telomeric DDR (DNA Damage Response), generates telomere replication stress phenotypes, and drives growth limiting responses such as senescence or apoptosis. In addition, because telomere maintenance strategies differ between tumours, it is important to understand how the same mode of shelterin disruption manifests across different cancer cell contexts. These questions define the practical research gap: connecting interface level shelterin disruption to robust, interpretable cellular and molecular outcomes in cancer cells. A further, thesis specific dimension of this gap concerns the availability and characterisation of chemical starting points. Our research group previously identified a reference compound, A822, based on an HTVS (High Throughput Virtual Screening) and SPR (Surface Plasmon Resonance)⁶⁰, which is considered a scaffold capable of disturbing telomere associated biology. The present thesis is built on derivatives of A822 rather than on a fully elucidated, mechanism defined clinical tool compound. Since the exact mechanism of action of A822 is still unclear, an important gap remains in understanding both what happens when the TRF1-TIN2 interaction is disrupted and how structurally related compounds behave across different biological experiments. It is also necessary to determine which results most consistently indicate telomere and shelterin related dysfunction. Therefore, the gap is both mechanistic, in linking TRF1-TIN2 disruption to cellular effects, and experimental, in confirming that the observed effects are truly related to telomere dysfunction.

Chapter 2

Aims and Objectives

This chapter presents the central hypothesis, aim, research questions, objectives, research strategy and significance of the thesis. The research focuses on small molecules modulating TRF1 and its interaction with TIN2. This interaction supports the organisation of shelterin at double stranded telomeric DNA and contributes to telomere replication and chromosome end protection.

2.1 Research hypothesis

The central hypothesis is that an in house synthesised compound, A822, and selected derivatives may bind TRF1 at or near the TIN2 binding region of the TRFH domain and interfere with TRF1-TIN2 supported shelterin organisation. Shelterin normally suppresses ATM and ATR linked DNA damage signalling at chromosome ends. Interference with the TRF1-TIN2 interaction is expected to weaken telomere protection and reduce the efficiency of DNA replication through telomeric repeats. The selected compounds are expected to cause slower replication fork progression, telomere associated DNA damage, abnormal telomere signals and chromosome end fusions.

The cellular response is expected to develop over time with treatment. Changes in telomere protection and replication are expected to alter DNA synthesis and cell cycle progression. Prolonged exposure may lead to senescence, mitochondrial dysfunction or apoptosis in a cell line dependent manner. These cellular changes are expected to reduce long term proliferation, migration, three dimensional spheroid growth, vascular branching and microtumour growth.

2.2 Aim of the research

The main aim of this thesis is to determine whether A822 and its newly synthesised derivatives can bind TRF1, disturb telomere protection and replication and limit cancer cell growth through a proposed effect on the TRF1-TIN2 axis. The research aims to relate the interaction of the compounds with TRF1 to telomere associated DNA damage, replication stress, structural telomere abnormalities and changes in cell fate. It further aims to determine whether these changes reduce long term proliferation, migration, three dimensional tumour growth, vascular branching and *in ovo* microtumour growth, and to relate the measured phenotypes to changes in gene expression and protein markers.

2.3 Research questions

This thesis addresses four linked research questions:

- (i) What cellular phenotypes arise when shelterin function is distorted in tumour cells, and how do these phenotypes relate to canonical telomere damage signalling outputs and telomere associated DNA damage foci?
- (ii) How does the disruption of TRF1 and its interaction with TIN2 influence telomere replication behaviour, proliferation capacity, and cell fate decisions such as senescence or apoptosis?

- (iii) Can newly synthesised compounds designed to perturb shelterin function induce measurable telomere deprotection and growth limitation in cancer cells, and do different chemotypes produce distinct response profiles?
- (iv) What changes at transcriptional levels and signalling networks follow compound induced shelterin dysfunction, and which of these changes best explain the observed phenotypes?

2.4 Research objectives

To answer these questions, the studies are organised around four linked objectives that move from defining the outcomes of shelterin dysfunction, to focusing on TRF1-TIN2 as an interaction node, to evaluating newly synthesised compounds, and finally to mapping pathway level changes induced by shelterin dysfunction.

Objective 1: *Define the role of shelterin dysfunction in cancer cells*

This objective establishes how disrupting the function of the shelterin complex alters cancer cell behaviour. This objective evaluates core readouts of telomere deprotection, including telomere associated DDR signalling, checkpoint activation, and changes in proliferation capacity. Because shelterin suppresses multiple DDR branches, the analysis considers both ATM and ATR linked responses and distinguishes telomere centred damage from generalised DNA damage where possible^{23,42}. The outcome is a defined set of cellular phenotypes and molecular markers that report shelterin dysfunction in proliferating tumour cells.

Objective 2: *Determine how TRF1 and TIN2 interactions contribute to tumour cell progression*

This objective focuses on the TRF1-TIN2 interaction as a specific architectural node within the shelterin complex. The study examines how interfering with TRF1 function and TRF1 retention through TIN2 affects telomere replication behaviour, telomere fragility related phenotypes, and telomere associated DDR activation. These molecular readouts are connected to functional outcomes relevant to tumour progression, including clonogenic growth and the ability to sustain proliferation under stress. The objective also sets the stage for interpreting compound effects by defining which outcomes are most consistent with disruption of the TRF1-TIN2 complex.

Objective 3: *Characterise the function of newly synthesised compounds designed to induce shelterin dysfunction*

This objective evaluates newly synthesised compounds as tools and potential leads for inducing shelterin dysfunction in cancer cells. The focus is on whether these compounds can reproduce key features expected from shelterin perturbation: induction of telomere associated DNA damage signalling, disruption of protective telomere states, and inhibition of sustained proliferation on therapeutically relevant timescales. Comparative analysis across compounds is used to determine whether different chemical structures produce distinct patterns of shelterin dysfunction, which is important for understanding structure activity relationships and for prioritising compounds for deeper mechanistic study.

Objective 4: *Map pathway level changes mediated through shelterin dysfunction in cancer cells*

This objective connects telomere centred perturbation to broader cellular programmes. The study maps changes in gene expression and pathway activity that follow compound induced shelterin dysfunction, with particular attention to pathways linked to senescence, apoptosis, cell cycle regulation, DNA repair, and replication stress. The rationale is that telomere deprotection should engage conserved DDR signalling and downstream programmes, but the balance between arrest and death can vary across tumour contexts. Defining these pathway level responses clarifies how shelterin

dysfunction translates into anticancer outcomes and identifies potential combination strategies that could enhance response durability.

2.5 Central research strategy

The central research strategy follows a planned sequence from compound selection to molecular and cellular analysis and complex tumour models. Our research group previously identified a small molecule inhibitor of the protein-protein interaction between TRF1 and TIN2. The compound, A822, originally obtained from the Asinex library under the identifier ASN 03579822, was selected through extensive computational screening and subsequently validated using biological and biophysical assays. The compound was resynthesised in our facility and is covered by patent WO2023182897A1 and was subsequently named as A822. The compound A822 serves as the reference scaffold for the derivative series (TRFiN). Initial cytotoxicity screening compares the compounds across cancer cell models and matched non-cancer cells when available. This stage is used to identify active derivatives and estimate selectivity. The most informative compounds are selected for time dependent experiments in HeLa and MCF7 cells. The parent compound and selected derivatives are compared throughout the research to examine how structural modifications affect binding, cytotoxicity, and cellular responses. The precise mechanism of action of A822 has not yet been disclosed. Because not all A822 related experimental components are available for use or disclosure across every assay context (including work performed by other researchers in our group and that is not yet published), the thesis employs assay specific positive controls that are selected for their ability to validate the technical performance of each biological experiment rather than for strict telomere specificity.

Computational and biophysical analyses form the first target related stage. Molecular docking examines whether A822 and selected derivatives can occupy the proposed TIN2 binding region of the TRF1 TRFH domain. Molecular dynamics simulations are used to assess the stability of predicted protein ligand contacts and to track changes in ligand position over time. SPR measures binding to purified TRF1 and provides kinetic and steady state estimates of affinity. Data from these analyses are used to select compounds for detailed cellular experiments.

The next stage examines whether the cellular response involves telomere dysfunction and altered DNA replication. Telomere associated damage is assessed from the relation between phosphorylated histone H2AX (γ H2AX) and TIN2 positive nuclear regions. Metaphase peptide nucleic acid fluorescence *in situ* hybridisation (PNA FISH) is used to identify single and double telomere loss, telomere signal doublets and chromosome fusions. Flow cytometry based FISH (Flow-FISH) is used to measure changes in bulk telomere fluorescence. The DNA fibre assay measures replication fork progression after sequential labelling with 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU). Cell cycle analysis with 5-bromo-2'-deoxyuridine (BrdU) and propidium iodide (PI) examines whether reduced DNA synthesis reflects slow S phase progression or accumulation in another cell cycle phase. These measurements are compared across treatment periods to examine the relationships among telomere abnormalities, replication fork progression, and cell cycle changes.

Cellular responses are monitored over multiple treatment periods to determine how telomere and replication stress affect cell fate. Live cell imaging, cell tracking and viability measurements record changes in proliferation and morphology. Senescence is examined by senescence associated fluorescence, cell morphology, loss of Lamin B1 and changes in cell cycle proteins. Mitochondrial membrane potential is examined using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), and mitochondrial organisation is measured to determine whether prolonged stress affects mitochondrial function. Apoptosis is assessed through Annexin V binding, 7-aminoactinomycin D (7-AAD) uptake, caspase-3/7 activity, poly (ADP-ribose) polymerase (PARP) processing and apoptosis inducing factor (AIF) redistribution. Clonogenic assays test the ability of treated cells to resume long term growth

after treatment, and migration assays measure changes in cell movement. The same experimental sequence is applied to HeLa and MCF7 cells to compare their responses to the selected compounds.

The strategy then moves from monolayer culture to models that retain more features of tumour growth. Three dimensional spheroids are used to examine whether growth suppression persists in a compact cell mass with cell cell contact and diffusion limited conditions. The chick chorioallantoic membrane model is used to study vascular branching and microtumour growth in a living tissue environment. Histological assessment of embryonic organs provides an initial examination of tissue injury under the tested conditions. RNA sequencing and pathway analysis are used to identify transcriptional changes after treatment. Protein measurements are compared with transcriptomic patterns and cellular assays to relate changes in gene expression and protein markers to DNA replication, cell cycle regulation, senescence, mitochondrial function, and apoptosis.

2.6 Significance and novelty

This research examines the TIN2 binding region of TRF1 as a possible site for anticancer drug development: many telomere directed approaches target telomerase, telomeric DNA, or DNA secondary structures. The present research studies focus on small molecules designed to interact with a protein binding region within the shelterin complex. TRF1 supports replication through double stranded telomeric repeats, and TIN2 connects TRF1 bound telomeric DNA with other shelterin components. Targeting this interaction may provide a direct way to alter telomere protection and replication in cancer cells.

The study examines telomere function using telomere associated DNA damage, metaphase telomere abnormalities, chromosome end fusions, replication fork progression and total telomere fluorescence. Time dependent experiments link these telomere and replication changes to cell cycle arrest, senescence, mitochondrial dysfunction, apoptosis, and loss of colony forming ability. The inclusion of A822 and several derivatives permits comparison of chemical structure, TRF1 binding, selectivity and biological activity. A comparison of HeLa and MCF7 cells examines whether the compounds produce similar telomere and replication effects in two cancer cell models with different cell cycle and cell death responses.

The novelty of the research lies in the combined examination of novel A822 derivatives using computational prediction, TRF1 binding, telomere specific measurements, DNA replication analysis, cell fate assays, three dimensional spheroids, the chick chorioallantoic membrane (CAM) model, RNA sequencing, and protein analysis. This research strategy connects compound design and TRF1 interaction with telomere function and cancer cell growth across molecular, cellular and *in ovo* models.

Chapter 3

Review of literature

3.1 Shelterin complex: composition, assembly, and core functions

Shelterin is a telomere specific protein complex that allows chromosome ends to remain stable without being recognised as DNA lesions⁶¹. It contains TRF1, TRF2, RAP1, TIN2, TPP1, and POT1. TRF1 and TRF2 bind double stranded DNA TTAGGG repeats through their Myb/SANT domains and form homodimers through TRF homology domains^{39,61}. RAP1 is recruited mainly by TRF2, while POT1 binds single stranded telomeric DNA. TPP1 binds POT1 and contributes to telomerase recruitment and processivity. TIN2 connects TRF1 and TRF2 with TPP1-POT1 and thereby links the double stranded and single stranded regions of the telomere^{19,61}. POT1 recognises the G-rich single stranded sequence through oligonucleotide/oligosaccharide binding folds and helps prevent inappropriate binding of replication protein A⁶². The locations and binding properties of these components allow shelterin to regulate telomere protection, replication, and length maintenance.

3.1.1 Protein-protein interactions within shelterin

Although each shelterin subunit has a clear binding niche at telomeres, telomere function depends strongly on the protein protein interactions that connect these subunits into a stable complex and shape which additional factors are recruited. Several interaction pairs are repeatedly described as the main interactions within shelterin: TRF1-TIN2, TRF2-RAP1, TIN2-TPP1, and TPP1-POT1. In addition, a TRF2-TIN2 connection provides further linkage that can stabilise TRF2 association and help integrate TRF2 driven end protection functions with the rest of the complex^{40,63}. These interactions are often treated as “core” because disrupting any one of them can break overall connectivity or mislocalise a significant component of the complex, with broad functional consequences at chromosome ends. The TPP1-POT1 interaction illustrates why this category matters. POT1 binds the single stranded telomeric overhang, but in cells, its stable telomeric localisation and proper engagement depend on its association with TPP1. Biochemical and structural studies have repeatedly supported this functional interdependence, indicating that POT1 is not simply an independent DNA binding unit but part of a coupled module whose telomeric function requires its partner protein. At the duplex end of the complex, TRF2-RAP1 plays a comparable organising role. RAP1 is recruited to telomeres primarily through TRF2, and this partnership influences how TRF2 behaves on telomeric DNA and how repair suppression is enforced. In particular, TRF2-RAP1 has been linked to the limitation of inappropriate repair outcomes, including processes related to homology directed repair^{64,65}. The TRF1-TIN2 interface is often described as particularly well defined because it reflects a specific docking interaction centred on the TRF1 TRFH region. Structural studies and deposited complex structures show that TRF1 recognises a defined TIN2 motif via a conserved surface within the TRFH domain, supporting the idea that TRF1-TIN2 represents a discrete PPI surface rather than a diffuse contact^{66,67}. This is an important conceptual point because it reinforces that parts of the shelterin complex are assembled through recognisable interfaces with defined sets of interacting amino acid residues. The TRFH region also functions beyond dimerisation. It acts as a recruitment platform, and similarities and differences between TRF1 and TRF2 influence which partner proteins each can bind. A widely cited study described a shared docking motif used by TRF1

and TRF2 that enables the differential recruitment of telomere associated proteins, illustrating how conserved structural logic can coexist with selective partner binding⁴⁹. This principle shared docking architecture combined with specificity helps explain why the TRF proteins act as central organisers of telomeric complexes rather than merely serving as just DNA binding proteins on TTAGGG repeats protecting DNA. TRF2 extends this organiser concept further. Structural and mechanistic studies have described TRF2 as a recruitment hub that recognises short peptide motifs and coordinates the binding of multiple telomere associated factors. This provides a mechanistic basis for why TRF2 influences several downstream outcomes beyond duplex telomeric DNA binding, including pathways linked to end protection and repair suppression⁶⁸. The main shelterin interactions and proposed assemblies are shown in Figure 3.1.

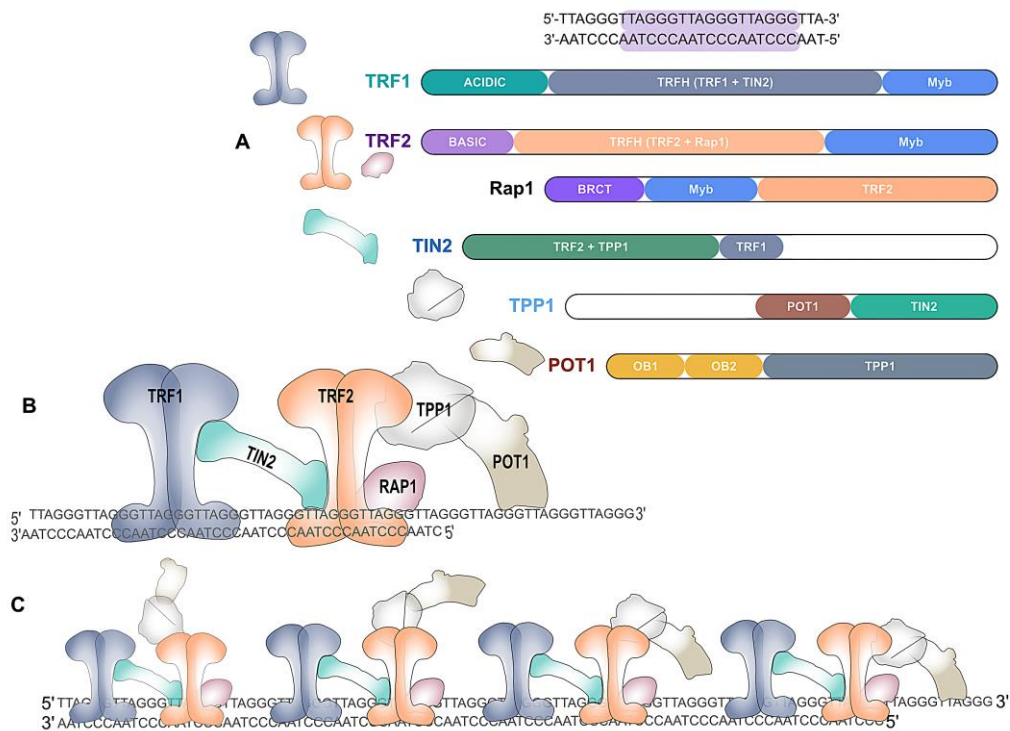


Figure 3.1. Shelterin complex. (A) The six identified shelterin subunits, showing their domain organisation, protein protein interactions, and DNA binding sites. POT1 can bind its target sequence at either the 3' terminus or an internal single stranded region. (B) Schematic representation of shelterin bound to telomeric DNA. For clarity, POT1 is depicted only at the binding site adjacent to the duplex telomeric region, although it can also bind at the 3' end. (C) Proposed shelterin assemblies and subcomplexes present at telomeres.

TIN2 has several interactions rather than acting only as a tether between subunits. In mammalian shelterin, TIN2 can bind TRF1, TRF2, and TPP1, and studies that combine structural and functional analyses emphasise that these multiple contacts allow TIN2 to stabilise the overall assembly and maintain the duplex to single strand bridge required for coordinated telomere regulation⁴⁰. Earlier mechanistic studies already highlighted the importance of the TIN2-TPP1 connection for higher order organisation and for integrating TRF2 linked functions with the POT1/TPP1 arm of shelterin. In experiments using recombinant proteins, TPP1 was shown to promote TIN2-TRF2 binding, supporting a cooperative assembly model rather than a collection of independent, isolated binary interactions⁶⁹. More recent studies extend this view by linking TIN2 not only to complex architecture but also to the control of telomerase. Evidence has been presented that TIN2 can cooperate with TPP1/POT1 to enhance telomerase processivity and strengthen recruitment, reinforcing the point that “assembly” at telomeres is closely linked to catalytic outcomes rather than only to physical localisation⁷⁰. In this context, distinguishing “core” from “accessory” interactions is functional because shelterin is not just a six protein cap; it is also a platform that coordinates many non-shelterin proteins that transiently associate with telomeres. The six shelterin components form the stable core, while

exposed surfaces on TRF1 and TRF2 recruit accessory factors involved in replication, end processing, and control of repair pathway choice. The TRFH docking platform is one route for this accessory recruitment, and a recurring theme in the field is that TRF1 and TRF2 use similar architectural principles yet recruit distinct partner sets, leading to different downstream outcomes^{49,68}. Therefore, the same structural elements that maintain shelterin integrity can also act as potential intervention points disturbing this integrity. Disrupting this interface could remove a core subunit and destabilise end protection, or block binding of a specific accessory factor, selectively weakening one telomere function without entirely collapsing the complex. Within this framework, the TRF1-TIN2 interaction occupies a strategic position: it is central enough to matter for shelterin architecture, yet sufficiently discrete to be considered a potential protein protein interaction target.

3.1.2 Coordination of end protection and telomere replication

Shelterin's core role is to prevent chromosome ends from triggering a complete DNA damage response (DDR) while still allowing the controlled processing required for telomere maintenance. Shelterin protects chromosome ends by blocking defined signalling enzymes and repair pathways that would otherwise treat telomeres as broken DNA. A widely used framework describes end protection in terms of two primary DDR branches: ATM, which is most often activated by double strand breaks, and ATR, which is commonly activated by single stranded DNA and replication stress. The Annual Review by Palm and de Lange describes how shelterin represses both ATM and ATR signalling and limits processing of chromosome ends by pathways such as non-homologous end joining (NHEJ) and homology directed repair (HDR), thereby insulating telomeres from the repair logic that is appropriate for internal DNA breaks but harmful at natural termini¹⁹. A key point in this framework is that repression is distributed across shelterin subunits rather than being the property of a single protein. Telomeres must simultaneously prevent several damaging outcomes. They must avoid end to end ligation, suppress recombination driven rearrangements, and limit persistent checkpoint signalling that would otherwise arrest proliferation. Mechanistically, TRF2 has been strongly linked to preventing ATM activation and blocking classical end joining at telomeres, whereas POT1 has been associated with preventing ATR activation by limiting RPA loading on the single stranded overhang and controlling how the 3' tail is presented to the damage signalling machinery. In functional terms, shelterin therefore partitions end protection tasks according to substrate: proteins that bind the duplex repeat sequence primarily address threats sensed at double stranded termini, while proteins that bind the single stranded overhang address threats that arise when single stranded DNA becomes exposed^{19,62}. These pathway dependent outputs are summarised in Figure 3.2.

TRF1 and TRF2 establish telomeric occupancy along the duplex repeat tract and provide platforms for the recruitment of additional factors. The POT1/TPP1 unit protects the single stranded overhang and helps coordinate regulated access by telomerase. RAP1 tunes TRF2 behaviour and contributes to recombination avoidance, while TIN2 stabilises the overall architecture, maintaining the integrity of the complex across repeated cell cycles. Replication is an essential part of this protection. Telomeric DNA must be duplicated and completed accurately during every S phase, and modern shelterin models increasingly treat replication as a core component of telomere protection. De Lange's 2025 review argues that shelterin is now understood as solving two linked problems: the classical end protection problem and the completion of telomeric DNA replication. In this model, shelterin acts as a recruitment platform for two telomere maintenance machines telomerase and the CST Pol α /primase complex which together replenish telomeric repeats and complete replication at chromosome ends. The review also provides a quantitative view of how much sequence is lost at telomeres per cell cycle and how fill in synthesis offsets this loss⁶¹. A key mechanistic point is that telomerase recruitment is associated mainly with the TPP1 side of the complex, whereas recruitment and regulation of CST Pol α /primase are linked to POT1, creating a division of labour that matches the DNA substrates each module engages⁶¹. Thus, the six subunit scaffold is not simply a static cap, but a controller that times when enzymatic activities can access the telomere terminus. In proliferating

cancer cells, where telomere duplication must keep pace with high division rates and where replication stress is often elevated, this role in telomere replication of shelterin becomes a major weakness, because even modest increases in telomeric replication failure can trigger persistent telomeric DNA damage signalling and threaten cell survival.

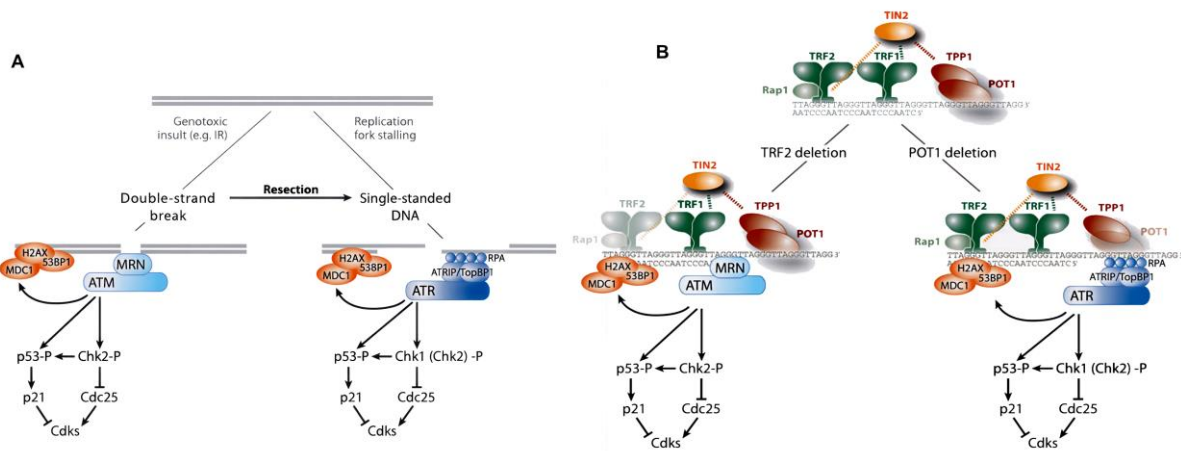


Figure 3.2. Shelterin mediated repression of DNA damage signalling. (A) Schematic comparison of the genome wide DNA damage response with the telomeric consequences of TRF2 or POT1 loss. (B) Overview of telomere specific damage response outcomes following deletion of TRF2 or POT1.

Within this system, the TRF1-TIN2 connection is relevant to drug development for several reasons. First, TRF1 binds the telomeric repeat tract duplex and has been closely linked to efficient telomere replication and the suppression of telomere fragility. This is especially relevant in rapidly dividing cancer cells, where telomeres are repeatedly challenged by replication pressure, and incomplete duplication can quickly translate into telomeric DNA damage signalling¹⁹. Second, the TRF1-TIN2 interaction is a defined interface that helps stabilise TRF1 within shelterin and connects TRF1 based telomeric occupancy to the rest of the complex. Structural mapping and studies focused on complex dynamics describe TRF1-TIN2 as a structured contact with identifiable interaction surfaces, supporting the view that it represents a tractable PPI target rather than a diffuse association⁶⁷. Third, TRF1 has progressed from a general telomere factor to a target that has been tested *in vivo*. Gracia et al. reported that therapeutic inhibition of TRF1 can suppress the growth of p53 deficient, KRAS driven lung tumours by inducing telomeric DNA damage, providing a direct functional link between TRF1 disruption and tumour control⁵⁴. In glioblastoma models, TRF1 upregulation has been reported, and brain specific Trf1 deletion has been shown to inhibit tumour initiation and progression while extending survival, supporting the view that TRF1 is a dependency in at least some aggressive tumour settings⁷¹. Safety is an important concern for any telomere directed strategy; Bejarano et al. directly examined this by showing that whole body Trf1 deletion in adult wild type and cancer prone mice caused only mild effects in normal tissues, while cells lacking Trf1 did not give rise to tumours. These findings suggest that TRF1 inhibition may provide a therapeutic window⁵⁵. Together, these observations on tumour sensitivity, combined with limited organismal toxicity in preclinical models, have advanced TRF1 from a conceptual telomere factor to a practical target candidate.

TRF1-TIN2 has gained further support from a recent chemical biology study directed at this interface. Casale and colleagues described “first in class” inhibitors of the TRF1:TIN2 interaction identified by fragment screening, with compounds that bind the TRF1-TRFH domain, displace TIN2 derived probes in biochemical assays, and expel TRF1 from purified shelterin preparations⁵⁶. Associated structural data further support that the interaction pocket is experimentally defined and can be engaged by small molecules as well as engineered peptides, directly addressing a standard limitation in protein protein interaction targeting (RCSB PDB: 9HHK)⁷². For cancer focused telomere targeting, this is important because TRF1-TIN2 lies at a junction where disrupting a single contact can

destabilise shelterin on the duplex repeat tract and induce telomeric DNA damage signalling without the delay associated with progressive telomere shortening. A probable sequence follows. If TRF1 cannot be stably retained at telomeres through its interaction with TIN2, both telomeric replication and local coordination of end protection become stressed. Under these conditions, telomeres accumulate DNA damage markers, checkpoint pathways are activated, and cell proliferation is restricted. Depending on the cellular context, this can also push cells toward apoptosis. Evidence from tumour models supports this sequence of events: interference with TRF1 function is sufficient to induce telomeric DNA damage, reduce tumour growth, and impair stemness associated properties, indicating that TRF1 is functionally important for tumour maintenance rather than merely a structural component of telomeres^{54,71}. Shelterin is therefore a compact but highly organised complex. It engages two DNA substrates: double stranded telomeric repeats and a single stranded overhang assembled through a limited set of dominant protein protein interactions; suppresses specific signalling and repair pathways that would otherwise convert telomeres into chromosome fusions or rearrangements; and regulates access to enzymatic activities required for end replication. The TRF1-TIN2 interaction is not a peripheral contact within this system. It represents a central interaction that links duplex repeat occupancy to the rest of the shelterin complex, and it is supported by experimental evidence for both therapeutic relevance and direct inhibitor discovery^{56,67}.

3.2 Shelterin architecture and structural organisation

3.2.1 Stoichiometry and multivalency at telomeres

Zinder and colleagues addressed these stoichiometry questions using a more controlled reconstruction approach. They purified full length recombinant human shelterin subunits, reconstituted defined subcomplexes and the complete shelterin ensemble, and then combined negative stain electron microscopy with cross linking mass spectrometry, native mass spectrometry, and mass photometry to map domain scale architectures and infer stoichiometries⁵². A central finding was that full shelterin, as well as TRF1 containing subcomplexes, adopted fully dimeric stoichiometries even in the absence of DNA. This suggests that a dimer based organisation is a default architectural state, established by protein assembly before telomeric chromatin is engaged⁵². The same study also reported extensive conformational heterogeneity across reconstituted complexes. Rather than being a technical complication, this heterogeneity appears to be an inherent property of shelterin. Flexible linkers and mobile domains allow the complex to sample a range of conformations, which is likely important for matching the changing geometries of telomeric DNA duplex sequences, junction regions, and end associated structures that vary across the cell cycle⁵². In functional terms, a heterogeneous and flexible complex can maintain occupancy while still accommodating replication, processing, and dynamic end structures. More recent live cell quantitative studies have further sharpened the question of how many shelterin molecules are present at telomeres and whether a single unified particle should be expected to dominate *in vivo*. Janovič and colleagues used endogenously tagged shelterin proteins in human cancer cells and quantified both total cellular abundance and telomere associated abundance. They reported that shelterin subunits are present at telomeres in roughly equal numbers and that telomeric chromatin is densely occupied by shelterin proteins. At the same time, they resolved the shelterin at telomeres into two non-overlapping subcomplexes rather than a single mixed unit at each binding site⁷³. This does not necessarily invalidate earlier abundance estimates. Instead, it supports a context dependent view in which equal representation on telomeric chromatin can coexist with unequal soluble pools in the nucleoplasm, and in which selective stability of particular modules under extraction can bias what is recovered biochemically. Under this interpretation, stoichiometry is best treated as conditional on the experimental context: what is assembled and maintained at telomeres in cells may differ from what survives purification yet still reflect real biology at chromosome ends.

3.2.2 Reconstitution studies and assembly routes

Reconstitution experiments are helpful because they clarify what is sufficient for shelterin assembly, what is only permissive, and what is limited by binding geometry. Lim, Zaug, and Cech addressed this by reconstituting a human shelterin “core” built around TRF2, TIN2, TPP1, and POT1, with the option to include RAP1, and then measuring stoichiometry using size exclusion chromatography coupled with multi-angle light scattering (SEC MALS) alongside independent fluorescence based quantification. The study identified an unequal 2:1 TRF2:TIN2 stoichiometry within the assembled complex. This indicates that one TRF2 homodimer associates with a single TIN2, rather than symmetrically binding two TIN2 molecules⁵⁰. They further showed that adding RAP1 preserved this TRF2:TIN2 ratio, while maintaining TRF2:RAP1 at 1:1 per TRF2 monomer that is, two RAP1 molecules per TRF2 dimer⁵⁰. Architecturally, this finding is important because it positions TIN2 as more than a simple two armed adaptor. Instead, TIN2 behaves as a hub that can impose asymmetry on an otherwise symmetric TRF2 dimer. One interpretation is that once one TIN2 bridges the relevant modules, recruitment of a second TIN2 may be sterically less favoured or functionally unnecessary under the conditions tested. In this sense, stoichiometry becomes a readout of physical constraints as much as of binding preference. The same reconstitution framework also clarified how DNA substrate geometry can influence where subcomplexes are likely to sit along telomeric chromatin. Lim and colleagues compared binding to purely duplex telomeric DNA, purely single stranded telomeric DNA, and junction like substrates that contain both regions. As expected, complexes containing POT1 showed a preference for telomeric ssDNA, while TRF2 containing complexes preferred telomeric dsDNA. However, the strongest binding was observed on substrates that mimic a telomeric ds/ss junction, and binding improved as the spacer length between duplex and single stranded binding sites increased⁵⁰. This supports the idea that shelterin assembly and positioning are tuned not only by which proteins are present, but also by the spatial arrangement of duplex and single stranded telomeric DNA regions that exist at chromosome ends.

They also reported that once the core complex is positioned on a ds/ss DNA junction substrate, it remains relatively stable even in the presence of excess telomeric dsDNA as a competitor. This provides a biochemical explanation for how a complex that contains both duplex and single stranded DNA binders can remain enriched near chromosome ends, even though telomeric duplex repeats are present in significant excess along the telomeric DNA sequence⁵⁰. In architectural terms, this positioning logic links stoichiometry to geography. The same components, when tethered in different ways, are likely to populate different telomeric microenvironments, which, in turn, determine which interfaces remain engaged for long periods and which contacts are sampled transiently. Single molecule and ensemble studies also support the view that shelterin does not need to form long cooperative filaments to occupy telomeric DNA repeats. Erdel and colleagues examined assembled shelterin and proposed a model in which shelterin complexes can exist in solution and locate telomeric DNA primarily through three dimensional diffusion. In their measurements, complexes could diffuse along non-telomeric DNA, but nucleosomes hindered this movement, arguing against extensive one dimensional sliding as the primary mechanism for finding and coating telomeric regions⁷⁴. This model is consistent with telomeric DNA being occupied by multiple independent complexes arranged in a “beads on a string” manner, rather than by a continuous lattice held together by strong shelterin shelterin cooperativity⁷⁴. Although these experimental systems simplify the native chromatin environment, the broader point holds for telomeres in cells: telomeric chromatin can be densely packed without requiring a rigid, repetitive higher order structure. This also aligns with the conformational heterogeneity observed in reconstituted human shelterin assemblies, which suggests that flexibility, rather than a fixed repeating architecture, is a defining feature of the system⁵².

3.2.3 Structural organisation on chromatin

Janovič and colleagues described two shelterin subcomplexes that occupy non-overlapping positions on telomeric chromatin: a TRF1-TIN2-TPP1-POT1 unit that associates tightly with telomeres, and a TRF2-RAP1 complex that binds more dynamically and has been proposed to support the recruitment of additional cofactors involved in chromosome end protection⁷³. Their data also link telomere dynamics to interaction strength. They note that binding between the TRFH like domain of TIN2 and the TRF2 hinge region is in the low micromolar affinity range and is substantially less stable than the TRF1-TIN2 interaction. This provides a molecular explanation for why the TRF2 centred module would exchange more rapidly at telomeres than the TRF1 centred unit⁷³. This division of labour has clear architectural implications. Rather than assuming that a single fully interlinked six subunit assembly occupies every telomeric binding site, telomeres may instead be tiled by modules that differ in residence time, coupling to the duplex repeat sequence, and capacity to recruit partner proteins. This type of modular occupancy is consistent with “beads on a string” models derived from biochemical and single molecule DNA binding experiments, where multiple independent complexes can densely occupy telomeric chromatin without requiring a rigid, fully cooperative higher order structure⁷⁴. Whole complex reconstitution studies show why the field has moved beyond a rigid view of shelterin as a single, uniform particle. Zinder and colleagues showed that reconstituted human subcomplexes and full shelterin, even when assembled from full length components and after truncation of some unstructured linker regions, still exhibit broad conformational variability. In their interpretation, this heterogeneity persists whether or not DNA is present, supporting the conclusion that flexibility is an intrinsic feature of shelterin rather than a structural state imposed by DNA binding⁵². From the perspective of telomeric chromatin, this is a reasonable outcome. Shelterin has to function on long repeat arrays, within nucleosomal packaging that differs from that of bulk chromatin, and in the presence of transient replication intermediates and end associated structures whose geometry changes throughout the cell cycle. A single locked conformation would struggle to meet these requirements. In contrast, a flexible ensemble of conformations can accommodate changes in spacing between binding sites and shifting local architecture, while still maintaining the key contacts needed for telomere protection and regulated access to the chromosome terminus when required. The structural basis of the TIN2 centred TPP1 and TRF2 contacts is shown in Figure 3.3.

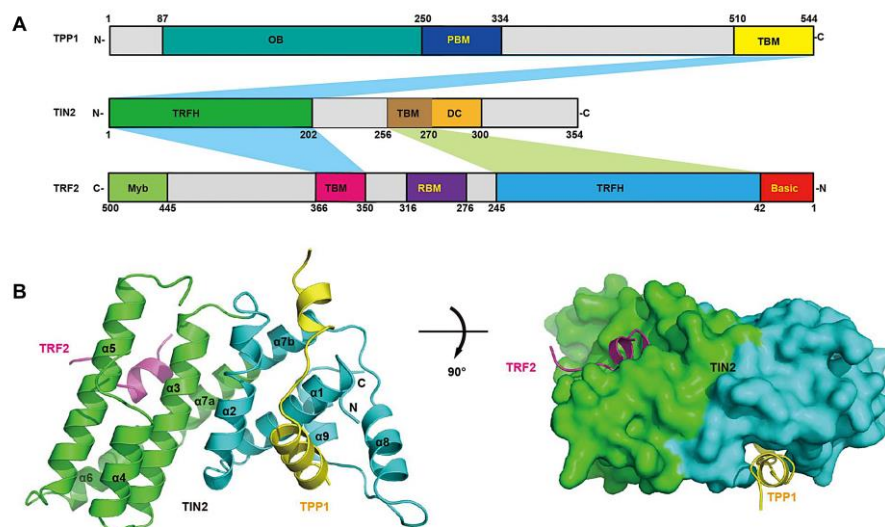


Figure 3.3. Structure of the TIN2-TPP1-TRF2 complex. (A) Domain architecture of TIN2, TPP1, and TRF2. OB, oligosaccharide binding fold; PBM (TPP1), POT1 binding motif; TBM (TPP1), TIN2 binding motif; TBM (TIN2), TRFH binding motif; DC, dyskeratosis congenita hotspot; Basic, N-terminal basic residue rich region; TRFH, telomeric repeat factor homology domain; RBM (TRF2), RAP1 binding motif; TBM (TRF2), TIN2 binding motif; Myb, C-terminal DNA binding domain. (B) Two orthogonal views of the TIN2_{TRFH}-TPP1_{TBM}-TRF2_{TBM} structure (PDB: 5XYF) shown as a cartoon (left) and TIN2_{TRFH} surface (right). TRF2 binding helical bundle is coloured green and TPP1 binding helical bundle is coloured cyan, with TPP1_{TBM} in yellow and TRF2_{TBM} in magenta.

Structural biology focused reviews have emphasised that the remaining questions about shelterin structure are not a simple consequence of limited effort, but instead reflect the same properties that make the complex functionally adaptable. Hu, Yan, and Nguyen note that high resolution structures are available for many individual shelterin domains and for short binding motifs that define key interfaces. In contrast, capturing the entire six subunit assembly at high resolution remains difficult because shelterin is flexible, multivalent, and strongly influenced by chromatin context and dynamic behaviour at telomeres⁷⁵. These structural features affect therapeutic design. Drug design often works best when it can exploit stable pockets and rigid interfaces, yet telomeric protein assemblies do not always provide such fixed targets. In practice, the most suitable sites are often motif based contacts at hub proteins, where a limited interface controls broader connectivity. This aligns with the mapped motif interactions of TIN2 with partner sequences and with the stoichiometric asymmetry revealed by core complex reconstitution, both of which point to discrete control points within an otherwise flexible system^{40,50}. Therefore, “targetability” has to be considered in architectural terms. It is not enough to ask whether a protein is important. One must also ask whether the relevant interface is engaged for long enough in cells, whether it is present in the chromatin state that matters for tumour growth, and whether its geometry can realistically be disrupted by a compound rather than being bypassed through alternative assembly routes. A quantitative perspective on shelterin has become increasingly important because telomere architecture is not only a matter of structure but also of populations. In current discussions of telomere maintenance, it is now common to consider that multiple shelterin assemblies can coexist at telomeres, and that functions such as telomerase recruitment, replication associated protection, and control of end structures can be distributed across modules rather than carried out by a single uniform complex. Broader reviews of human shelterin and telomerase biology have synthesised this problem in quantitative terms, including how assembly state and stoichiometry intersect with telomerase regulation and telomere maintenance outputs⁷⁶. For this study, this distinction is important. Once shelterin is treated as a modular, dimer based, and compositionally flexible architecture, then targeting a specific protein protein interface particularly hub centred contacts such as those involving TIN2 becomes a rational strategy. In this framework, a compound need not completely dismantle telomeres. Even shifting the equilibrium between more stable and more dynamic subcomplex states can have measurable consequences for telomere replication, telomere associated DNA damage signalling, and cancer cells' ability to sustain proliferation.

3.3 Telomere protection and control of DNA damage responses

3.3.1 *The chromosome end protection problem*

Telomeres have an unusual position in the genome. They are natural chromosome termini, yet the defining chemical feature of a telomere, which is a DNA end resembles the defining feature of a double strand break. If cells treated telomeres as accidental breaks, checkpoint pathways, nucleases, and ligases would be engaged in ways that compromise chromosome integrity. The chromosome end protection problem includes a set of damaging outcomes that must be prevented continuously: inappropriate checkpoint activation, end to end joining, excessive end processing, and recombination driven rearrangements. One of the clearest ways this problem has been defined is not by focusing on a single preferred readout, but by experimentally removing the telomeric protein coat and mapping the complete set of responses that follow. In a particularly informative genetic strategy, the entire shelterin complex was functionally removed from mouse telomeres by conditional deletion of TRF1 and TRF2, thereby collapsing shelterin occupancy and exposing chromosome ends to the default DNA damage surveillance and repair machinery⁴². The outcome was not a single telomere phenotype, but a broader inventory of threats that telomeres normally suppress. These included activation of both ATM and ATR signalling, engagement of end joining pathways beyond the classical route, and additional forms of telomeric processing that can be difficult to detect when only one shelterin subunit is perturbed⁴². This whole complex removal approach also addresses a common interpretive trap in

telomere biology. Single subunit deletions often trigger an early dominant outcome that masks other pathways. For example, if telomeres fuse rapidly, the chromosome ends that would otherwise undergo resection or recombination are no longer available as free substrates, and downstream processing routes become difficult to observe because the substrates have been removed from the system. Checkpoint activation can also distort which pathways are observed. If a strong checkpoint response arrests the cell, repair activities that act mainly in cycling cells become under represented, simply because the relevant substrates are no longer being generated under normal replication conditions. By forcing telomeres into a shelterin free state, Sfeir and de Lange therefore framed end protection as the combined suppression of multiple DDR signalling branches and repair pathways that would otherwise act at chromosome ends, rather than as the consequence of one protective interaction⁴². De Lange's later mechanistic synthesis reinforced this view by detailing how shelterin prevents telomeres from inappropriately activating DNA damage signalling and double strand break repair pathways²³. Together, these sources provide a valuable anchor for a literature review: telomere protection is best described in terms of pathway control and concrete outputs such as fusions, resection, recombination, and checkpoint signalling rather than as a general statement that telomeres are "capped."

Telomere protection is dynamic throughout the cell cycle and as DNA structural states change. Some models emphasise end hiding, particularly through t-loop formation, because sequestration of the terminus could physically limit access of factors such as Ku70/80 and the MRE11-RAD50-NBS1 complex (MRN), thereby reducing both NHEJ and ATM activation. Ruis and Boulton discuss how end hiding models can be aligned with genetic observations and why cellular state and experimental context influence which protection mechanisms dominate in different settings²⁴. For a functional chapter, the key implication is that end protection cannot be reduced to a single static structure. Telomeres are replicated, processed, and remodelled, and each stage alters which intermediates exist and which DDR sensors could, in principle, bind to chromosome ends. Because shelterin is a protein complex, its ability to resolve the end protection problem depends on its organisation and on how it shapes the interface between telomeric chromatin and the DNA repair machinery. Structural and mechanistic overviews increasingly emphasise that loss of shelterin function exposes telomeres to multiple DDR pathways, and that the six subunit arrangement enables suppression of several distinct pathway outputs at the same chromosomal locus⁷⁵. This multi-output control matters because telomeric DNA is not an inert substrate. Telomeric repeats can adopt unusual DNA structures; they are embedded in chromatin, and telomeres can also experience lesions within the repeat sequence, not only at the terminal end. This point is illustrated by studies in which double strand breaks are introduced within telomeric repeat arrays. Telomere internal breaks can activate ATM signalling in S phase yet still be tolerated and repaired, showing that "repair at telomeres" is not uniformly blocked; it depends on whether the lesion is located at the natural chromosome terminus or inside the telomeric sequence⁷⁷. That distinction strengthens the logic of end protection. The most stringent suppression is required at the natural end, because end joining or inappropriate processing there can directly alter chromosome topology and generate fusions. When shelterin is present, telomeres remain compatible with long term proliferation by maintaining DDR signalling and repair within controlled limits. When shelterin is removed, chromosome ends become substrates for the same pathways that repair accidental breaks elsewhere in the genome. For these reasons, the end protection problem is best understood as the combined action of the DDR and repair pathways, which must be restrained at telomeres, along with the consequences when restraint fails. Canonical shelterin functions can therefore be mapped in a pathway output manner, by asking which subunit or submodule suppresses which specific outcome, such as checkpoint signalling, end joining, resection, or recombination^{23,42}.

3.3.2 Control of ATM, ATR, NHEJ, HDR, and end processing

A key genetic result showed that the main telomere checkpoint pathways can be controlled separately, rather than acting as a single, uniform “telomere damage response.” Denchi and de Lange demonstrated that dysfunctional mammalian telomeres can activate both ATM and ATR, and that two shelterin components act as the primary repressors of these pathways: TRF2 suppresses ATM, whereas POT1 prevents ATR activation⁴¹. One point from that study is especially relevant for telomere fusions. The authors found that signalling through either ATM or ATR is required for efficient NHEJ at deprotected telomeres⁴¹. This directly links checkpoint signalling to repair competence at chromosome ends, indicating that ATM/ATR repression is part of repair pathway control, not merely the suppression of a marker response. TRF2’s canonical protective role is closely tied to limiting ATM activation and preventing classical end joining at chromosome termini. Recent imaging and DNA topology studies have helped translate this idea into physical mechanisms. Super resolution imaging provided direct evidence that TRF2 promotes t-loop formation and supported the view that t-loop remodelling can explain reduced ATM signalling and reduced NHEJ when TRF2 is present⁷⁸. In parallel, research focused on telomeric DNA topology argued that TRF2 dependent DNA wrapping creates a protective topological state that inhibits both ATM activation and NHEJ, and that RAP1 can act as a backup barrier when topology based protection is weakened⁷⁹. These studies connect the common statement “TRF2 suppresses NHEJ” to measurable telomere structures and DNA states, rather than leaving it as an assumption. NHEJ control also involves interactions with major end joining factors. Ku70/80 is a major DNA end binding protein for c NHEJ and is often viewed as a threat to telomeres because it can support end fusion. However, genetic experiments show that Ku can also act protectively at dysfunctional telomeres by limiting other inappropriate repair events. Celli and de Lange reported that Ku70 stimulates the fusion of dysfunctional telomeres yet protects telomeres from recombination between sister telomeres, illustrating that the same repair factor can be harmful in one setting and protective in another⁸⁰. Mechanistically, TRF2 associated modules can inhibit NHEJ very early, at the stage of DNA dependent protein kinase (DNA-PK) engagement. Bombarde et al. described a “double protection” model in which TRF2/RAP1 blocks classical NHEJ mediated end fusion at the initial DNA-PK end binding/activation step, while DNA-PK can counteract a Lig4 dependent process that would otherwise promote telomeric end fusion under certain conditions⁸¹. This places TRF2 linked protection at the earliest step of end recognition and helps explain why changes in TRF2 output can rapidly shift the fusion phenotype (Figure 3.4A).

RAP1’s contribution can also be demonstrated in direct biochemical assays. In a human extract system, a RAP1/TRF2 complex inhibited non-homologous end joining of telomeric DNA substrates, supporting a direct protective role for RAP1 when tethered to TRF2⁸². Thus, TRF2-RAP1 is not only an organisational pairing at telomeres but also a functional unit that suppresses end joining. TRF2-RAP1 also restricts homology directed repair. End fusions are the most obvious telomere catastrophe, but telomeres also need protection from homology driven repair because the repeat arrays provide abundant homology and can invite inappropriate recombination. In mammals, RAP1 has a clear role here. Rai and colleagues provided evidence that TRF2-RAP1 is required to prevent telomeres from engaging in HDR, positioning RAP1 as a central anti recombination factor at telomeres rather than a dispensable accessory⁶⁴. In pathway terms, RAP1 is best framed as a suppressor of recombination based outcomes at telomeres, rather than a primary checkpoint suppressor. POT1-TPP1 controls ATR signalling and overhang protection. ATR repression at telomeres is linked to how single stranded telomeric DNA is handled, particularly at the duplex to single strand junction and the 3’ overhang. The prevailing model is that POT1 competes with RPA for ss telomeric DNA and prevents ATR activation by keeping RPA off the overhang. Structural studies support this by showing that human POT1 protects the telomeric ds ss junction in a manner consistent with limiting RPA loading and ATR signalling⁸³. Control of the single stranded DNA overhang is also linked to replication stress. Functional studies show that POT1 inactivation induces telomere replication stress, activates an ATR

dependent DDR, and increases telomere fragility, thereby linking ATR signalling at telomeres to replication linked defects rather than to end exposure alone⁸⁴. Therefore, ATR at telomeres can be driven by ssDNA exposure, replication problems, or both, and POT1 constrains these states by controlling how ss telomeric substrates are presented during and after replication. TPP1, often introduced as POT1's binding partner and a telomerase recruitment factor, also has a defined protective role in limiting ATR linked processing. Kibe et al. showed that TPP1 blocks an ATR mediated resection mechanism at telomeres, linking the POT1/TPP1 unit not only to signalling suppression but also to control of end processing that would otherwise generate long ssDNA sequences and amplify ATR responses⁸⁵. TPP1 therefore restricts resection behaviour that feeds ATR at telomeres, rather than serving only as a general support factor for POT1 (Figure 3.4B).

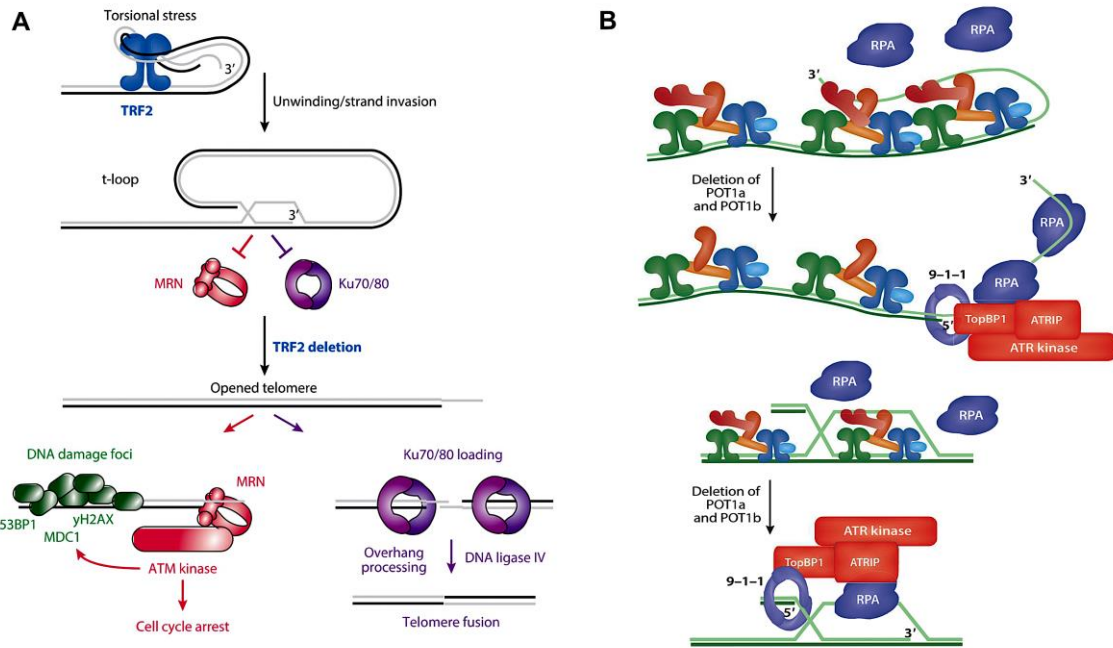


Figure 3.4. Shelterin mediated repression of telomeric DNA damage signalling. (A) TRF2 suppresses ATM activation and classical NHEJ by promoting t-loop formation, which limits end access by MRN and Ku70/80. Loss of TRF2 enables MRN dependent ATM signalling with recruitment of DNA damage factors at telomeres, and allows Ku loading to initiate c NHEJ via DNA ligase IV. (B) POT1a loss (or POT1a/POT1b loss) activates ATR at telomeres through RPA coated ssDNA and the TopBP1-ATRIP-ATR axis.

End processing also depends on the TRF2-Apollo pathway. Apollo, also known as SNM1B or DCLRE1B, is a 5' exonuclease recruited to telomeres through TRF2. A protected telomere is not an end that never gets processed; it is an end that is processed in a controlled way and then re-protected. This can be seen most clearly at the leading strand of telomeric DNA after replication, where regulated 5' processing is required to generate a 3' G-overhang. This nuclease is one of the best characterised processing factors in this setting. Wu et al. showed that Apollo contributes to G-overhang maintenance and protects leading end telomeres, and that these telomeric functions depend on Apollo's TRF2 interaction and nuclease motifs⁸⁶. Lam et al. provided a complementary mechanistic view by showing that SNM1B/Apollo is required to protect leading strand telomeres against NHEJ mediated repair and that its exonuclease activity contributes to overhang generation at newly replicated leading strand telomeres⁸⁷. These findings show that telomere protection requires controlled nuclease activity rather than complete exclusion of nucleases. Canonical telomere protection allows limited and timed nucleolytic activity to form the mature end structure while suppressing uncontrolled degradation and ligation. Telomere protection is also influenced by cell cycle dependent repair choice. Repair control at telomeres is strongly shaped by cell cycle context, both because the end is presented differently at different stages and because access to repair pathways changes with the cell cycle. Konishi and de Lange examined cell cycle control of telomere protection and NHEJ and showed that TRF2 function

and end joining engagement are modulated across the cell cycle, indicating that “protection” includes constraints on when NHEJ is allowed to act at telomeres⁸⁸. This cell cycle dependence may be overlooked when phenotypes are measured in asynchronous populations. A telomere that is protected in G1 can still be transiently vulnerable in S phase if replication intermediates are mishandled. Conversely, an end that is vulnerable in G1 might not show the same outcomes in S/G2 if repair access and processing differ. Organising shelterin’s canonical roles by pathway provides a set of testable expectations. Perturbing TRF2 should bias telomeres toward ATM signalling and NHEJ type outcomes, influenced by t loop/topology control and early steps in DNA-PK engagement^{78,81}. Perturbing POT1/TPP1 should bias telomeres toward ATR signalling and ATR linked processing defects, particularly under replication stress⁸³. Weakening TRF2-RAP1 output should increase susceptibility to recombination type outcomes and change how telomeric substrates are handled by HDR⁶⁴. Removing the complete shelterin ensemble exposes all of these pathways at once, which is why it remains the benchmark experiment for defining what telomeres must suppress to preserve chromosome identity⁴².

3.4 TRF1 structure, telomere replication, and regulation

3.4.1 TRF1 domain organisation and interaction surfaces

TRF1 (TERF1) is a double stranded telomeric repeat binding factor that sits on TTAGGG arrays as a homodimer. By doing so, it sets the physical and biochemical context in which replication and repair proteins encounter telomeric chromatin. TRF1 is often described as having two main functional blocks: an N-terminal TRFH domain that mediates dimer formation and provides a recruitment platform, and a C-terminal Myb/SANT DNA binding domain that makes sequence specific contacts with DNA telomeric repeats. Structural studies on isolated TRF1/TRF2 TRFH domains showed that these dimerisation modules are largely α -helical and adopt a “horseshoe like” fold. Dimerisation is essential because it is the state in which TRF1 becomes competent for stable occupancy of duplex telomeric DNA and for multivalent engagement along long repeat sequence³⁹. At the other end of the protein, NMR and related analyses of the TRF1 DNA binding domain in complex with a DNA telomeric sequence clarified how the Myb like fold reads bases within the repeat, with a recognition mode centred on a AATCCC [or TTAGGG] element and a binding geometry that resembles, but is not identical to, that of classical Myb proteins^{89,90}. Reduced TRF1 occupancy can arise because reduced telomeric occupancy can arise either from weakening of sequence recognition at the Myb/SANT domain or from disruption of the dimeric architecture that positions two DNA binding domains on the repeat array. The TRFH domain is also where TRF1 is regulated through short linear motifs in partner proteins. A clear example is the TRF1-TIN2 connection, in which TRF1 recognises a conserved docking motif via a defined surface on TRF1-TRFH. This docking logic is shared with TRF2, even though the downstream recruitment outputs differ⁴⁹. The existence of a shared docking motif, supported experimentally and structurally, helps explain how TRF1 and TRF2 can recruit overlapping sets of accessory proteins while still forming functionally distinct subcomplexes at telomeres⁴⁹.

These structural features show that TRF1 function is not limited to double stranded DNA binding. Its full length architecture appears suited to maintaining binding across chromatin substrates and long repeat arrays, consistent with the need to retain an adequate density of TRF1 on telomeres throughout S phase⁹¹. It also helps reconcile why telomeres with the same sequence can behave differently when TRF1 abundance changes. Telomeric repeats may be plentiful, but TRF1 occupancy across those repeats can drop below a functional threshold at long telomeres or under stress conditions, producing replication associated fragility⁹². In cancer, these structural features link TRF1 to stress and signalling pathways. TRF1’s interaction surfaces link telomeres to stress and signalling pathways on timescales that do not require telomere shortening. TRF1 occupancy is tuned by partner proteins and by post translational modifications that influence residence time, solubility, and retention on chromatin. When these layers shift, the telomere can move from a relatively inert chromatin

domain to a substrate that triggers ATR signalling during S phase or accumulates telomeric DNA damage foci, even when telomere length remains within a non-critical range^{93,94}. TRF1 is therefore a cancer relevant target because it provides a route to induce telomere dysfunction on a shorter therapeutic timescale than strategies that rely on gradual telomere erosion.

3.4.2 TRF1 in telomere replication and replication stress

The most explicit functional definition of TRF1 in mammalian cells came from a study that treated telomeres as replication barriers rather than as passive chromosome end labels. Sfeir and colleagues showed that TTAGGG arrays behave like difficult to replicate DNA and exhibit replication dependent defects that resemble those of common fragile sites. In that setting, TRF1 is required for efficient telomere replication, and TRF1 deletion triggers ATR activation in S phase together with a “fragile telomere” phenotype scored on metaphase spreads⁹³. Fragile telomeres are measured concretely: telomere FISH reveals multiple telomeric signals (MTSs), where a single chromatid end shows MTSs that are consistent with under replication or abnormal processing at the telomere⁹⁵. This is a simple cytogenetic readout, but it has become central because it connects molecular problems at forks to a phenotype that scales with replication stress and with the proteins that resolve it. Later studies refined this view by separating what TRF1 does to support fork progression from how it keeps checkpoint signalling contained. Zimmermann et al. showed that TRF1 helps cells negotiate repeat associated replication problems by recruiting Bloom syndrome helicase (BLM) and, in parallel, using the TPP1/POT1 module to repress ATR signalling during telomere replication. The study is notable because it splits TRF1 output into two protective tracks: one focused on the physical movement of the replication machinery through telomeric DNA, and another on limiting a checkpoint response that can destabilise telomeres if it persists⁹⁶. This distinction is relevant for tumour cells, where a telomere can experience fork stress without immediate end fusions yet still accumulate telomeric DDR signals that reduce proliferation, increase apoptosis, or alter cell cycle progression. Related phenotypes are observed in mouse models of TRF1 dysfunction. Telomere fragility and telomeric damage foci can occur together with checkpoint activation and, over time, translate into degenerative phenotypes and altered cancer susceptibility, with outcomes depending on the status of p53 and other checkpoints⁹⁴. Thus, TRF1 loss can push tissues toward growth failure or instability and tumour risk, depending on barrier integrity.

Replication stress at telomeres is now viewed as more than simple fork stalling on repeats. Telomeric chromatin can promote secondary structures, impose topological constraints, and create conflicts with transcription or processing events, and TRF1 helps maintain a telomeric environment where these obstacles do not become catastrophic. Porreca et al. combined inducible TRF1 loss with telomere focused proteomics and concluded that TRF1 supports S phase telomeric DNA synthesis, thereby preventing illegitimate recombination and chromatin remodelling later in the cell cycle. They also formalise telomere fragility scoring using MTSs and connect TRF1 loss to a shift in the telomeric protein composition⁹⁵. Mechanistically, adjacent studies suggest that fragile telomeres can also arise through stress mediated break induced replication (BIR). Yang et al. used chromosome orientation fluorescence *in situ* hybridisation (CO-FISH) and related assays to support a model in which BIR promotes fragile telomere formation, providing a route by which telomeric replication problems spill into recombination associated DNA synthesis rather than being resolved cleanly by canonical fork restart⁹⁷. These ideas matter for cancer settings because BIR, mitotic DNA synthesis (MiDAS), and recombination pathways can be unusually active in tumour cells and may buffer the loss of one protective layer until a second layer is compromised⁹⁸. Recent models have revisited TRF1 linked telomeric replication stress using more detailed replication structure logic. Vauris and colleagues argue that TRF1 limits lagging strand telomere fragility through pathways that involve fork reversal and restart processing. Their studies implicate additional partners including transcription factor IIH (TFIIH) and show that alternative bypass routes, such as DNA primase polymerase (PrimPol) mediated repriming, can rescue specific fragility outcomes when fork reversal is impaired⁹². This

model provides a mechanistic explanation for why TRF1 density matters: low TRF1 “coverage” at long telomeres could increase the likelihood that stalled forks are processed less effectively, leading to visible fragility signals⁹². It also aligns with how telomeric replication stress is usually measured in practice. Studies commonly combine cytogenetic and molecular readouts, including scoring of MTSs in metaphase telomere FISH, telomere dysfunction induced foci (TIFs) in interphase, ATR/checkpoint kinase 1 (CHK1) activation during S phase, and single molecule replication assays that monitor fork progression through telomeric DNA⁹³. An additional layer, important in cancer biology, is that ATR can suppress telomere fragility and recombination. As a result, the phenotype produced by TRF1 loss depends on how much ATR signalling is allowed versus how much it is blocked⁹⁹. This is relevant in tumour contexts where ATR inhibitors are considered clinically. Weakening TRF1 function increases telomeric replication stress, and inhibiting ATR at the same time can shift a manageable defect into persistent telomeric damage and proliferative failure^{93,99}.

3.4.3 Regulation of TRF1 and its relevance to cancer therapy

TRF1 is one of the more tightly regulated shelterin components at both the protein stability and telomere binding levels, and these regulatory layers create possible targets for intervention. A classic example is the tankyrase-TRF1 axis. Tankyrase was initially identified as a telomere associated poly (ADP-ribose) polymerase that binds TRF1 via ankyrin repeats, placing TRF1 under PARP family control, which can influence its retention at telomeres¹⁰⁰. In human cells, tankyrase overexpression promotes telomere elongation, consistent with a model in which poly ADP ribosylation (PARylation) driven release of TRF1 increases telomerase access¹⁰¹. The cancer relevance of this pathway became clearer when Seimiya et al. showed that tankyrase 1 (TNKS1) inhibition in human cancer cells enhances telomere shortening when combined with telomerase inhibition and accelerates cell death, positioning tankyrase as a co-target that acts through TRF1 rather than by directly inhibiting telomerase¹⁰². More recent studies extend this idea into stress settings by showing that tankyrase 1 can be recruited to damaged telomeres via TRF1 and can PARylate TRF1 after damage, suggesting a damage responsive version of the same regulatory circuit¹⁰³. These findings show that TRF1 is not only structural; it is a regulated substrate whose post-translational modification can change telomeric occupancy, end processing, and downstream DDR outputs. TRF1 abundance is also controlled by ubiquitin mediated proteolysis, providing a second set of enzymatic regulatory pathways. Chang et al. reported that TRF1 can be ubiquitinated and degraded after its loss from telomeres, suggesting a coupling between chromatin dissociation and proteasomal turnover¹⁰⁴. Specific ubiquitin ligases have been identified, including SCF complexes using the F box protein FBX4 as the substrate recognition component. FBX4 targets TRF1 for ubiquitin dependent degradation and can strongly influence TRF1 abundance and telomere length control¹⁰⁵. Structural studies resolved the basis of selective TRF1 ubiquitination by SCF-FBX4, providing a molecular rationale for how TRF1 is recognised and why changes in TRF1 conformation or modification state may alter degradation efficiency¹⁰⁶. RING finger LIM domain interacting protein (RLIM; RNF12) has also been described as a TRF1-interacting ubiquitin ligase that modulates telomere length homeostasis through TRF1 proteolysis, offering an additional route to shift TRF1 turnover without involving FBX4 directly¹⁰⁷. Additional regulators affect this pathway; U2 small nuclear RNA auxiliary factor 2 (U2AF65), for example, has been reported to stabilise TRF1 by interfering with the TRF1-FBX4 interaction, showing that non canonical regulators can influence telomere protein abundance through ubiquitin dependent regulation¹⁰⁸. From a therapeutic perspective, these findings show that enzymes that add or remove ubiquitin, or proteins that gate access to those enzymes, can shift TRF1 levels at telomeres without directly blocking DNA binding.

Phosphorylation provides another major control layer, placing TRF1 at the interface between telomeres and oncogenic signalling. A prominent example is phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) regulation. Mendez Pertuz et al. showed that chemical inhibitors of PI3K and

AKT reduce TRF1 telomeric foci and induce telomeric DNA damage and fragility, linking a major cancer pathway to a measurable change in telomere protection through TRF1 regulation¹⁰⁹. Sánchez Vázquez et al. extended this by demonstrating that AKT dependent extracellular cues regulate TRF1 phosphorylation, affecting TRF1 stability and telomere binding and influencing tumourigenic potential. This is important because it suggests that TRF1 can respond to microenvironmental signals upstream of PI3K/AKT rather than being determined solely by intrinsic telomere length¹¹⁰. Other kinases act in more cell cycle restricted contexts. Polo like kinase 1 (PLK1) mediated phosphorylation increases TRF1 telomeric DNA binding and peaks during mitosis, consistent with a model in which mitotic kinase activity helps set TRF1 chromatin association at a time when telomeres must be managed for proper sister telomere resolution¹¹¹. CDK1 has also been implicated in TRF1 phosphorylation (including phosphorylation at T371), and evidence suggests that phosphorylated TRF1 can be recruited to DNA damage sites and that cell cycle kinase control influences TRF1's subnuclear behaviour and chromatin association¹¹². ATM/ATR signalling interacts with TRF1 regulation in distinct ways. Tong et al. reported that ATM and ATR regulate recruitment of TRF1 to DNA damage sites and promote phosphorylation dependent loss of TRF1 from telomeres. This is relevant because loss of TRF1 from telomeres can increase fork stalling and amplify replication stress phenotypes¹¹³. A site specific ATM output was described by McKerlie et al., in which ATM phosphorylates TRF1 at S367, reducing TRF1 telomeric binding and placing canonical DNA damage signalling directly upstream of TRF1 release from telomeres¹¹⁴. CK2 mediated phosphorylation has also been linked to telomere length homeostasis by regulating TRF1 levels at telomeres, reinforcing the idea that phosphorylation is not a single switch but a collection of modifications that tune stability and residence time¹¹⁵. Under oxidative stress, additional kinase control emerges: NIMA related kinase 7 (NEK7) was reported to interact with and stabilise TRF1 at telomeres, phosphorylate TRF1, and thereby reshape TRF1 regulation and telomere integrity¹¹⁶.

Beyond phosphorylation, prolyl isomerase regulation also connects to TRF1. Peptidyl prolyl cis trans isomerase NIMA interacting 1 (Pin1) has been described as regulating TRF1 stability and telomere function through phosphorylation dependent conformational changes, reinforcing the recurring pattern that enzymatic control does not require direct DNA binding to alter telomere behaviour¹¹⁷. Finally, TRF1 phosphorylation has been linked to the choice of telomere maintenance pathway. Ho et al. reported that phosphorylation at T271 influences telomerase dependent telomere maintenance and affects the formation of ALT associated promyelocytic leukaemia (PML) bodies, suggesting that TRF1 can influence telomere maintenance logic beyond strictly telomerase positive settings¹¹⁸. The case for TRF1 as a cancer relevant target is supported by *in vivo* studies showing that TRF1 loss or inhibition can restrain tumour growth. García Beccaria et al. demonstrated that genetic ablation of Trf1 impairs growth of p53 deficient KRASG12V driven lung cancer and increases survival, with the effect linked to telomeric DNA damage, apoptosis, reduced proliferation, and cell cycle arrest. Importantly, the strategy was described as telomere length independent, meaning it does not require a long erosion phase to become effective⁵⁴. In glioblastoma models, Bejarano et al. reported that Trf1 deletion increased telomeric DNA damage and reduced proliferation and stemness. Pharmacological reduction of TRF1 through PI3K/AKT pathway inhibition produced similar effects in human glioblastoma cells and reduced tumour sphere formation and xenograft growth from patient derived glioblastoma stem like cells⁷¹.

An important concern for telomere targeting is toxicity in normal tissues, since telomere dysfunction is traditionally linked to stem cell failure. Pathway level evidence also points to clinically relevant connections: inhibition of PI3K/AKT reduces TRF1 telomeric foci and increases telomeric fragility, linking commonly targeted signalling pathways to telomere destabilisation via TRF1 and suggesting an indirect way to exploit TRF1 dependency¹⁰⁹. Clinical association studies suggest that TRF1 alterations or expression patterns can correlate with disease behaviour in specific contexts,

although these signals are not uniform across tumour types and should be interpreted with the same caution as many telomere related biomarkers. For example, a prostate cancer analysis reported TRF1 amplification in a subset of castration resistant samples and linked TRF1 alteration patterns to survival measures in specific cohorts¹¹⁹. In renal cell carcinoma cells, TRF1 silencing has been reported to reduce proliferation, promote apoptosis, and induce cell cycle arrest, supporting the idea that some tumours show functional sensitivity to TRF1 depletion even outside engineered mouse models¹²⁰. In this thesis, TRF1 represents more than a single “block telomere maintenance” concept. It is a replication facilitator at complex repeats, a platform for recruiting factors that restrain replication associated checkpoint activation, and a protein whose telomeric residence can be tuned through PARylation, ubiquitination, and phosphorylation. Each of these layers offers a possible pharmacological target, depending on context and the extent to which a tumour depends on telomere integrity^{93,100,106,110}.

3.5 TIN2: scaffold function, isoforms, and telomere regulation

TIN2 (encoded by TIN2) first entered telomere biology as a TRF1-interacting factor identified through interaction based approaches, and was subsequently found to be directly at telomeres, where depletion or mutation affected telomere length regulation in human cells¹²¹. From the beginning, the importance of TIN2 was not that it binds telomeric DNA itself, but that it organises other telomeric proteins into a stable working unit. This point became clearer when studies showed that TRF1 and TRF2 do not act as isolated modules *in vivo*. Instead, they can be physically and functionally coupled through TIN2, allowing telomeric chromatin to preserve length control inputs even when one duplex binder is perturbed¹²². Biochemical studies supported this view by describing a higher order telomere associated complex containing TRF1, TRF2, RAP1, POT1, TPP1 (referred to as PTOP or related names in early research), and TIN2. In this model, TIN2 is required to bridge assemblies rather than binding telomeric DNA directly¹²³. Reconstitution experiments then added an important constraint: stable higher order assembly requires both TIN2 and TPP1 to support bridging between the TRF1 and TRF2 subcomplexes, and weakening the TIN2-TPP1 connection reduces the robustness of the assembled complex and its telomeric targeting⁶⁹. In parallel, the linkage that positions the single stranded end binding machinery was clarified when PIP1 (later recognised as TPP1) was shown to connect POT1 to the TIN2/TRF1 complex, providing a concrete mechanism for how duplex bound factors can control POT1 loading and telomerase access¹²⁴. TIN2 is therefore best considered a scaffold rather than a passive linker: it sits at the centre of multiple binding obligations that together determine how telomeres are assembled and how their ends are interpreted. TIN2 is also not a single fixed entity. Alongside the commonly discussed short and long isoforms (TIN2S and TIN2L), additional isoform complexity has been reported, including a longer form initially highlighted for features such as nuclear matrix association¹²⁵. Isoform differences became more than annotation when experiments compared isoforms for functional outputs related to telomerase action and telomere length, and a third isoform (reported as TIN2M in later studies) further expanded the range of TIN2 isoforms while retaining telomeric localisation⁷⁰. The presence of multiple isoforms does not mean each isoform encodes a completely different biology. Instead, it suggests that cells can tune scaffold behaviour binding strength, residence time, or interaction balance while still preserving the essential bridging role. In cancer, the same scaffold protein can be used in different states: one state may favour stable end protection during normal proliferation, while another may support telomerase positive expansion with altered telomere length set points.

3.5.1 Interaction motifs, structural organisation, and disease associated mutations

At the molecular level, TIN2 behaves as a hub because it harbours distinct interaction surfaces for multiple partners and can coordinate their engagement. Structural studies clarified this by solving the N-terminal TIN2 architecture in complex with partner motifs, showing how TIN2 can bind TRF2- and TPP1 derived elements and support stable assembly logic⁴⁰ (Figure 3.5). This is relevant to therapy

because hub proteins can have separable interfaces. Perturbing a single interface may not mimic complete shelterin loss; it may instead produce selective defects, such as reduced recruitment of POT1/TPP1, while leaving other shelterin interactions partly intact. This is relevant to drug development because telomere dysfunction may be induced by disrupting a specific protein-protein interaction without completely removing the shelterin complex from telomeres. This modularity is visible experimentally. In some systems, telomere protection functions that generally depend on a TRF1 interaction can be bypassed by enforcing a TRF2 tethered configuration, showing that a TRF2 linked TIN2/TPP1/POT1 module can still execute core protective tasks when it is loaded efficiently at telomeres¹²⁶. Mouse genetics also supports the idea that tethering is functionally important within the complex: POT1/TPP1 mediated end protection requires TIN2 based tethering to suppress inappropriate ATR signalling at telomeres¹²⁷. A focused analysis strengthened this conclusion by showing that direct TPP1 binding to TIN2 is required for POT1a/b function in telomere protection, linking a specific protein-protein interaction to a defined end protection output¹²⁸. Together, these studies place the TIN2-TPP1 axis among the sensitive routes by which end protection and signalling control can fail. TIN2 also shapes telomerase facing behaviour.

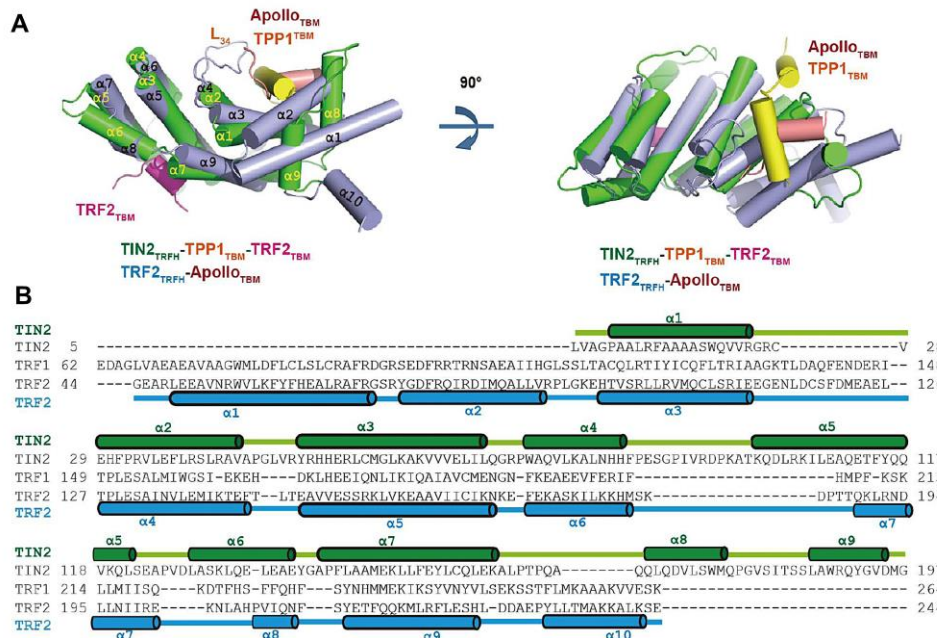


Figure 3.5. Structural similarity of the TIN2_{TRFH} domain to the TRFH domains of TRF1 and TRF2. (A) Overlay of TIN2_{TRFH}-TPP1_{TBM}-TRF2_{TBM} with TRF2_{TRFH}-Apollo_{TBM}, showing that TPP1_{TBM} and Apollo_{TBM} occupy analogous surface pockets on TIN2_{TRFH} and TRF2_{TRFH}. (B) Structure guided sequence alignment of TIN2_{TRFH}, TRF1_{TRFH}, and TRF2_{TRFH} with secondary structure elements indicated (α helices as cylinders; loops as lines).

In human cells, telomerase recruitment is not solely a function of telomerase reverse transcriptase (TERT)/telomerase RNA component (TERC) expression; it also depends on establishing the appropriate docking environment at the telomere end. Experiments have shown that a TIN2 anchored arrangement supports telomerase recruitment via TPP1¹²⁹, making the scaffold relevant to telomerase activity even though TIN2 is not an enzyme. This fits with the established role of POT1-TPP1 in regulating telomerase processivity¹³⁰ and with biochemical data showing that POT1-TPP1 can enhance processivity by controlling primer dissociation and translocation¹³¹. In that framework, TIN2 is more than a passive connector. It can influence processivity outputs in a TPP1/POT1 dependent manner, and this activity has been tested across TIN2 isoforms, suggesting that TIN2 actively contributes to a telomerase permissive microenvironment rather than merely ensuring localisation⁷⁰. Because telomerase action is usually tightly timed and limited to a subset of telomeres per cycle, even moderate changes in TIN2 dependent assembly could shift elongation probability across a proliferating cell population. Finally, TIN2 links telomere length control to post-translational

regulation through tankyrase. TIN2 can modulate tankyrase 1 PARP activity in the TRF1 telomere length control pathway, anchoring TIN2 directly to a circuit that affects TRF1 residence and telomere length outcomes¹³². In telomerase positive tumours, changing TIN2 dependent assembly can alter telomerase access and telomere stability without directly inhibiting the telomerase enzyme itself, and without waiting for long term telomere shortening.

Human genetics provides strong evidence that TIN2 integrity is essential for telomere homeostasis. Pathogenic TIN2 mutations are well established in telomere biology disorders such as dyskeratosis congenita. Early studies reported recurrent mutations in conjunction with very short telomeres, supporting the idea that partial disruption of the scaffold can drive systemic telomere failure^{132,133}. Additional cohorts reinforced the link and expanded the clinical phenotype range, while still pointing to marked telomere shortening as a standard feature¹³⁴. Functional studies suggest that some disease linked TIN2 variants act as separation of function alleles, with altered interactions relevant to telomerase regulation and telomere length outcomes rather than simply causing nonspecific protein loss^{135,136}. These disease associated findings are relevant for oncology because they imply that modest quantitative changes in TIN2 activity/function can shift telomere set points. That concept is made explicit in a study proposing that TIN2 can act as a haploinsufficient tumour suppressor by limiting telomere length and enforcing a proliferative barrier¹³⁷. Mammalian genetic studies also show that reduced TIN2 can promote ALT associated phenotypes in embryonic stem cell contexts, suggesting that severe TIN2 disruption can open access to recombination based telomere states rather than stable telomerase governed maintenance¹³⁸. Although these findings were not obtained in a cancer model, they indicate that TIN2 levels and structure can influence the telomere maintenance mechanisms available to a cell. TIN2 also contributes to telomeric DNA organisation and sister telomere behaviour through interactions that extend beyond its established role in telomere end protection.

Studies on telomere cohesion showed that TRF1 and TIN2 interact with the stromal antigen 1 (SA1) containing cohesin complex, and this linkage affects sister telomere association and resolution, linking a scaffold protein to chromosome behaviour in S/G2 and mitosis¹³⁹. Related studies demonstrated that cohesin variant requirements differ between telomeres and centromeres, supporting the idea that telomeres use specialised cohesion logic¹⁴⁰. Mechanistically, a heterochromatin protein 1 (HP1) binding site within TIN2 has been reported to be necessary for sister telomere cohesion, again pointing to a defined motif on the scaffold as a determinant of a specific chromosome level phenotype¹⁴¹. Single molecule and imaging approaches have further examined how TIN2 contributes to telomeric DNA compaction and DNA-DNA bridging together with TRF1. These experiments showed differences between isoforms in compaction, bridging and emphasised TRF1-TIN2 as an architectural pair rather than merely a recruitment module⁶⁷. Complementary studies focusing on TRF2-TIN2 similarly describe TIN2 as an architectural protein that supports TRF2 mediated DNA interactions, consistent with a broader role in organising telomeric DNA states that are important for protection and replication¹⁴². More recent quantitative biophysics suggests that TRF1, TIN2, and SA1 can act synergistically in telomeric DNA compaction and that assembly order influences compaction outcomes. This indicates that telomeric architecture is sensitive to stoichiometry and assembly route, not only to whether components are present¹⁴³.

3.6 The TRF1-TIN2 interaction: structural basis and regulation

The TRF1-TIN2 interaction is often drawn as a single line in shelterin diagrams, but it acts as an important regulatory interaction that can reshape telomeric protein distribution, local DNA organisation, and signalling outcomes when replication stress increases. TRF1 is built to recognise duplex DNA TTAGGG arrays as a homodimer, and the structural basis for this lies in the TRFH dimerisation domain, a stable, largely α -helical dimer that also exposes a conserved recruitment

surface used by partner proteins³⁹. Early biochemical and DNA bending studies established that TRF1 binds duplex telomeric DNA as a dimer and can impose curvature on the repeat sequence, a property that becomes more relevant when a scaffold such as TIN2 promotes multivalent assemblies¹⁴⁴. The C-terminal Myb/SANT DNA binding domain provides sequence discrimination, and structural analyses of Myb DNA complexes explain how DNA bases in the telomeric repeat are read in the major groove, explaining why TRF1 occupancy depends on repeat content and spacing⁸⁹. TRF1 function can therefore be disrupted by loss of repeat recognition at the Myb domain or by loss of stable dimer based engagement at the TRFH domain. Even within duplex repeats, TRF1 is not locked into a single rigid geometry. TRF1 can bind bipartite telomeric sites with substantial spatial flexibility, consistent with a model in which the dimer can accommodate variable half site spacing across chromatinised repeats while still maintaining stable residence¹⁴⁵. This flexibility is relevant at telomeres because repeat arrays are long, nucleosomal organisation differs from that of bulk chromatin, and the distances between usable binding configurations can vary across the array. TIN2 acts as a scaffold that couples TRF1 rich regions of duplex telomeric DNA to other shelterin modules and to accessory factors that tune higher order organisation. TIN2 can stabilise multivalent binding and bridging states, which may alter how telomeric DNA is compacted and how the replication machinery interacts with the repeat sequence. The broader shelterin context is important because TRF recruitment surfaces evolved under the requirement to build a protected telomere end state without triggering repair. Shelterin is therefore considered a dedicated complex rather than a loose collection of end binding proteins⁶³. Mechanistically, the TRFH domain is not only a dimerisation unit but also a docking platform, and an important structural finding for TRF1-TIN2 was the “shared docking motif” model. TRF1 uses a conserved surface in its TRFH domain to recognise TIN2. In contrast, the analogous surface in TRF2 is directed toward other partners. This explains how two related duplex binders can recruit different accessory factors despite having similar overall architecture⁴⁹. It also makes the TRF1-TIN2 interface attractive for inhibitor design because it is a PPI organised around a defined surface and peptide recognition logic rather than diffuse electrostatic interactions. The same docking surface is also used for recruiting other TRF1 partners, so perturbing it can simultaneously weaken multiple telomere supportive interactions.

TIN2 was initially characterised as a telomere length regulator that binds TRF1 and affects telomere homeostasis in cells, providing an early functional basis for why disrupting TRF1-TIN2 can matter beyond general disruption of the complex¹²¹. *In vitro*, TIN2 can stimulate interactions between telomeric DNA sequences, consistent with scaffold driven clustering or bridging that is not produced by TRF1 alone. This was later supported by a single molecule study showing that TIN2 increases the stability and complexity of TRF1 mediated DNA-DNA bridging events¹⁴⁶. The consequences of TRF1-TIN2 binding are therefore not limited to recruitment; they extend to how repeat arrays are compacted, looped, and bridged within the same telomere or between different telomeric DNA molecules. These DNA states can influence replication fork progression, the probability of fork stalling, and the need for restart pathways, all of which connect directly to telomere fragility phenotypes. Isoform diversity provides an additional level of regulation. A longer isoform (often termed TIN2L) and other forms have been reported, with differences in localisation tendencies and interaction behaviour that raise the possibility that TRF1-TIN2 assemblies can be tuned by isoform composition as well as by post-translational regulation¹²⁵. Biochemical studies have also shown that TIN2 can cooperate with TPP1/POT1 to stimulate telomerase processivity, indicating that TIN2 can influence telomere elongation decisions in addition to organising duplex repeat binding⁷⁰. Once TIN2 bridges TRF1 bound duplex arrays to the POT1/TPP1 module, shifts in TIN2 occupancy driven by TRF1 abundance, TRF2 dynamics, or binding site availability can be translated into measurable changes in end maintenance and in overhang protection. The importance of the TIN2-TPP1 connection for building robust complexes was shown by interaction and reconstitution studies demonstrating that TPP1 and TIN2 promote stable telomeric assembly⁶⁹. Structural studies then defined how TIN2

can serve as a platform for binding multiple partners, including TPP1 and TRF2, providing interface logic that supports models in which competition and simultaneous binding can both occur depending on context and conformational state⁴⁰.

Available evidence supports conditional assembly rather than one static shelterin complex. Quantitative biophysical approaches have driven this shift by providing binding constants, kinetics, and stoichiometric constraints that allow direct comparison of assembly models. One example is the use of quantitative peptide TRF domain measurements to compare how TRF1 and TRF2 engage TIN2 and Apollo derived motifs, allowing competition to be measured quantitatively¹⁴⁷. Importantly, competition is not always symmetric. Studies dissecting TRF1, TRF2, TIN2, and TPP1 interactions support a regulated exchange model in which TRF1 can promote TRF2 disengagement from TIN2, while TPP1 can remodel TIN2 associated states⁵¹. These exchange behaviours align with models that describe shelterin as a dynamic end protection machine. In that context, altering TRF1-TIN2 connectivity can have broad consequences once end processing and checkpoint thresholds are considered²³. Mechanistic studies of shelterin function provide the background needed to understand why disrupting a single PPI can expose pathways that are usually restrained, especially under replication stress, when telomeres transiently resemble damaged DNA¹⁹. Human genetic findings support the same conclusion: pathogenic TIN2 mutations in telomere biology disorders show that relatively small perturbations of this scaffold can collapse telomere function *in vivo*, arguing that TIN2 dependent assemblies are required structures rather than being redundant¹³³. Recent single molecule studies have examined how TRF1-TIN2 alters telomeric DNA organisation. Single molecule and imaging approaches, including atomic force microscopy (AFM), total internal reflection fluorescence microscopy (TIRFM), and DNA tightrope assays, show that TIN2 stabilises TRF1 mediated bridging and promotes the formation of larger multiprotein assemblies on long telomeric substrates. The same platforms also show that regulatory inputs, such as tankyrase activity and TPP1 derived fragments, can reduce or reshape bridging, providing an explanation for how partner availability and enzymatic control feed into telomeric architecture⁶⁷. Complementary studies focusing on TRF2 show that TIN2 can also facilitate TRF2 mediated cis and trans interactions on telomeric DNA, supporting the broader view that TIN2 is an architectural component whose scaffold function is expressed through multiple duplex binding factors¹⁴².

When TRF1, TIN2, and other factors, such as SA1, are combined, telomeric architecture becomes sensitive to assembly order. Quantitative approaches, including nanochannel based studies, have shown that maximal DNA compaction can depend on the route by which components meet the substrate. This indicates that stoichiometry and binding measurements are not sufficient; assembly history can shape the resulting state¹⁴³. At the scale of the complete shelterin system, evidence that shelterin forms dimeric assemblies with substantial structural plasticity helps explain why TRF1-TIN2 occupancy may vary across telomeric chromatin and why multiple subcomplexes can coexist instead of collapsing into a single fixed arrangement⁵². Structural reviews emphasise that the current understanding is dominated by discrete interface modules rather than a single monolithic complex, which supports inhibitor strategies that target one interface while leaving other shelterin contacts intact⁷⁵. Direct chemical evidence also supports this approach. Small molecule inhibitor studies targeting the TRF1-TIN2 interaction have been reported, explicitly exploiting the defined nature of the TRF1 docking surface and peptide recognition logic for screening and optimisation, and grounding the approach in earlier evidence that TRF1 perturbation can restrain tumour growth⁵⁶. Competition at this interface is not limited to TRF1, TRF2, and TPP1. It also involves regulatory inputs that modify TRF1 residence time and partner binding, and those inputs can change which binding states dominate at telomeres. In this context, tethering and recruitment studies that mapped how shelterin components connect such as a study showing that TPP1/POT1 tethering and recruitment depend on a defined linkage architecture help explain why weakening TRF1-TIN2 can propagate into altered POT1

positioning and, hence, reduced ATR suppression at telomeres¹²⁷. Recent cell based and mechanistic studies further indicate that TRF1-TIN2 interaction states can be remodelled *in vivo* through partner driven mechanisms. A study examining interactions among TRF1, TRF2, TIN2, TPP1, and POT1 supports the view that this interface sits within a regulated network rather than functioning as a single binary binding event⁵³. Together, these studies show that TRF1-TIN2 is supported by multiple evidence types structures defining docking surfaces, biochemical assays quantifying binding and exchange, single molecule data showing altered DNA organisation, isoform studies indicating adjustable scaffolding, and disease genetics demonstrating scaffold fragility so it can be treated as a defined molecular target. Inhibition at this interface may induce telomere specific replication stress or end protection defects, without requiring direct inhibition of telomerase.

3.7 Anticancer strategies targeting shelterin and TRF1-TIN2

Efforts to target telomere biology in cancer therapy have faced an important limitation: direct telomerase inhibition generally requires prolonged treatment before telomeres become critically short and drive cells into crisis, as discussed by Sekaran and colleagues¹⁴⁸. Later reviews of agents targeting telomere dynamics have similarly noted that many strategies depend on gradual telomere shortening rather than inducing rapid telomere dysfunction¹⁴⁹. In contrast, shelterin focused strategies aim to convert telomeres into an acute damage signal without waiting for long term shortening. *In vivo* evidence was obtained from lung cancer models, where pharmacologic interference with TRF1 telomeric binding induced telomeric DNA damage and suppressed growth of established KRAS/p53 driven tumours⁵⁴. Studies in glioblastoma further supported this idea using both genetic and pharmacological approaches. TRF1 upregulation has been reported in mouse and human glioblastoma (GBM), and brain specific *Trf1* deletion inhibited tumour initiation and progression. Pharmacological reduction of TRF1 levels and its association with telomeres produced similar cellular effects in human GBM cells⁷¹. This body of evidence also motivated focused drug discovery commentary that identified telomeres as possible therapeutic targets in GBM rather than merely as biomarkers¹⁵⁰. Because any telomere directed approach raises an immediate safety question, studies that explicitly address toxicity have been influential. Follow up physiological studies in *Trf1* deficient mice described changes in body composition and metabolic readouts, reminding that systemic TRF1 loss can produce measurable organism level effects even when overt lethality is absent¹⁵¹. These results helped shift the field toward the idea that telomere disruption can be used as a rapid source of telomeric replication stress and DNA damage rather than a strategy that depends on gradual telomere shortening. In rapidly dividing tumours, even a modest increase in uncapped or poorly replicated telomeres can accumulate over a few cell cycles and translate into checkpoint activation and loss of clonogenic growth.

Across these studies, on target activity has been monitored using a relatively consistent set of telomere readouts. A common first step is to document displacement or reduction of TRF1 at telomeres by immunostaining, often paired with telomeric DNA detection. Downstream, telomere dysfunction induced foci (TIFs) are assessed by co-localisation of DDR markers with telomeric signals, building on the initial use of tumour protein p53 binding protein 1 (53BP1) co-localisation with telomeres from Takai et al.¹⁵² and a later methodological study that standardised antibody/telomere co-detection and scoring criteria for routine TIF quantification¹⁵³. Replication associated outputs have been especially informative for TRF1 directed interventions, because TRF1 loss results in fragile telomeres, which are visible as aberrant telomeric signals on metaphase chromosomes. The mechanistic link between stalled replication at telomeric repeats was established in the seminal study showing that mammalian telomeres resemble fragile sites and require TRF1 for efficient replication⁹³. Subsequent studies connecting TRF1 dysfunction to recombination prone states and break induced replication provided a route to understanding why TRF1 perturbation can also drive genome instability signatures beyond telomeres when cells attempt to survive replication stress⁹⁵. These

features improve mechanistic interpretation because a response accompanied by clear TIF induction and fragile telomere accumulation is easier to attribute to telomere stress than an endpoint measured only as reduced proliferation. At the same time, selectivity remains an important issue. TRF1 perturbation can engage ATR linked replication stress programmes that interact with p53 status, checkpoint signalling capacity, and replication/repair capacity in different tumour contexts, creating opportunities for combination therapy while also complicating resistance predictions. Importantly, TRF1 linked phenotypes often appear early, during ongoing replication, thereby reducing the delay between target engagement and a measurable response and making these assays practical for short treatment windows in models.

Chemical strategies have therefore expanded beyond direct interference with TRF1 DNA binding toward approaches that shift shelterin architecture through defined protein-protein contacts, with particular interest in the TRF1-TIN2 interface. A key reason is that this interface is structurally defined and suitable for structure guided inhibitor development. The observation that TRF1 and TRF2 share conserved docking surfaces in their TRFH domains for differential recruitment, including TIN2 recognition by TRF1, established the 'shared docking motif' framework that continues to guide inhibitor design⁴⁹. Later structural analyses showed that TIN2 itself contains a TRFH like interaction platform and can bind partner motifs from TPP1 and TRF2 cooperatively, which helps explain how changing a single contact can remodel others rather than disrupting only one binary interaction⁴⁰. Quantitative exchange models have also supported conditional competition among TRF1, TRF2, and TPP1, depending on whether TPP1 has engaged TIN2 and expanded its binding capacity⁵¹. When chemical probes are evaluated, an apparent TRF1-TIN2 inhibitor could indirectly alter TRF2 behaviour or TPP1/POT1 recruitment by reshaping the network rather than cleanly disrupting a single binary interaction. Direct chemical evidence that the TRF1-TIN2 interface can be targeted came from a fragment screening approach reporting small molecules that bind TRF1(TRFH), displace a TIN2 derived probe, and expel TRF1 from purified shelterin preparations⁵⁶. In parallel, peptide and peptidomimetic strategies have been proposed to modulate TRF1/TRF2 shelterin functions by designed interference with shelterin PPIs, with emphasis on profiling cellular responses (damage markers, viability, and telomere phenotypes) to separate target related effects from generic peptide toxicity⁵⁷. A third route has been to reduce TRF1 occupancy indirectly by targeting upstream regulators. One study linked PI3K/AKT signalling to TRF1 phosphorylation, TRF1 stability, and TRF1 telomere binding, and reported that PI3K or AKT inhibition reduces TRF1 telomeric foci and increases telomeric damage/fragility, including in patient derived breast cancer xenografts responding to PI3K α inhibition¹⁰⁹. A later report emphasised extracellular cues upstream of AKT that regulate TRF1 phosphorylation and connect telomeric effects to tumourigenic potential in engineered systems¹¹⁰.

It remains uncertain how best to demonstrate on target engagement *in vivo* with minimal ambiguity. Loss of TRF1 foci by immunofluorescence is informative, but it can reflect protein downregulation, epitope masking, or displacement. Telomeric TIF scoring is sensitive but can also report indirect replication stress, and fragile telomere quantification is mechanistically informative yet labour intensive and prone to differences in scoring thresholds across laboratories. In this context, reconstitution and stoichiometry studies that define how shelterin subcomplexes assemble, and how multiple pathways can influence telomerase processivity, help with the interpretation of biochemical displacement experiments in intact chromatin⁵⁰. Structural studies that identify protein-protein interaction interfaces as potential drug targets also indicate that the multiple and dynamic interactions within these complexes can limit the cellular effects of compounds with low binding affinity⁷⁵. Resistance to shelterin directed treatment has not been examined thoroughly. Loss or inhibition of TRF1 function can induce telomeric DNA damage responses that restrict tumour cell proliferation. However, cancer cells may adapt through changes in DNA damage checkpoint signalling, increased tolerance to replication stress, or changes in telomere maintenance mechanisms. These possible responses remain important considerations when developing therapeutic approaches that target

TRF1⁵⁸, and clinical reviews emphasise that even when target engagement is documented, selective pressure can reshape malignant populations over time¹⁵⁴. Within shelterin focused strategies, pathway based modulation introduces additional selectivity concerns because upstream pathways, such as PI3K/AKT, have extensive non-telomeric roles.

In contrast, direct TRF1 or TRF1-TIN2 inhibitors may allow clearer assignment of the mechanism but still face potency, delivery, and off target hurdles typical of PPI and chromatin associated targets. The tankyrase-TRF1 axis provides a clear example of this limitation. Tankyrase binds and PARylates TRF1 and can thereby influence telomere elongation¹⁵⁵, while TIN2 modulates tankyrase activity within the TRF1-mediated telomere length control complex¹³². However, the broad non-telomeric functions of tankyrase limit its suitability as a telomere-selective anticancer target, an issue also recognised in early studies proposing tankyrase as a telomere-directed therapeutic target in cancer cells¹⁵⁶. For telomerase directed drugs, this has been illustrated by clinical experience with agents such as the telomerase inhibitor Imetelstat, where biological activity can be observed but overall outcomes still reflect tumour type, treatment schedule, and the capacity of clones to adapt under pressure¹⁵⁴.

3.8 Non-canonical and extra-telomeric roles of shelterin proteins

3.8.1 *Extra-telomeric functions of RAP1*

RAP1 is usually described as the TRF2 bound adaptor within shelterin, and that description is accurate for telomeres: mammalian RAP1 is recruited by TRF2 and contributes to end protection by limiting inappropriate repair reactions at chromosome ends, including the repression of NHEJ when partnered with TRF2⁸². However, RAP1 became difficult to classify as “only telomeric” once genetic and functional assays showed that loss of RAP1 can produce phenotypes that a simple telomere deprotection model cannot explain. began to frame RAP1 as an adaptor with parallel telomeric and non-telomeric functions¹⁵⁷. One clear non-telomeric branch is inflammatory and stress signalling: Teo and colleagues identified telomere independent RAP1 as an I κ B kinase (IKK) complex adaptor that modulates NF- κ B dependent transcription, separating this activity from telomere binding and positioning Rap1 as a cytoplasmic/nuclear signalling component rather than a strict chromosome end factor¹⁵⁸. That study matters for oncology because NF- κ B circuitry directly influences survival, cytokine programmes, and microenvironmental interactions; it also matters for drug development because perturbing RAP1 or its interactions may change inflammatory thresholds even when telomeres remain structurally capped. A second, independently supported extra-telomeric branch is metabolic gene regulation. In RAP1 deficient mice, Paula Martínez and colleagues reported obesity and metabolic disturbances linked to altered transcriptional programmes, suggesting that RAP1 acts extratelomerically to regulate gene expression in metabolic tissues¹⁵⁹. In a closely aligned study, Yeung et al. linked RAP1 to metabolic control and protection against obesity, again pointing to a transcriptional mechanism rather than telomere uncapping as the main cause¹⁶⁰. These metabolic findings are often discussed as “physiology,” but in cancer, they become relevant because tumour cells and the tumour bearing host share nutrient and hormone environments, and because inflammatory metabolic coupling can shape tumour progression.

Mechanistically, several lines of evidence support the idea that RAP1 can influence chromatin and transcriptional states without being restricted to telomeric repeats. Genome scale localisation studies reported RAP1 occupancy beyond telomeres and argued that telomeric proteins can appear at selective internal genomic sites, raising the possibility that RAP1 contributes to gene regulation either through direct chromatin association or through partnership with transcriptional regulators¹³⁵. This indicates that if RAP1 is engaged in transcriptional regulation via protein complexes, then cancer associated RAP1 effects may depend more on cell specific interacting proteins than on telomere length alone. Consistent with that, later studies linked RAP1 to chromatin modifying assemblies, such as TIP60/p400, in cell state regulation, providing a concrete example of RAP1 acting through an

epigenetic complex rather than through the shelterin architecture¹⁶¹. Telomeric and non-telomeric functions are not always clearly separated. A genetic interaction between RAP1 and telomerase deficiency revealed an additional role for RAP1 in telomere maintenance under particular constraints, suggesting that RAP1 biology can shift depending on telomere status and cellular background¹⁶². In cancer focused discussions, RAP1 is now commonly treated as a multifunctional factor whose tumour relevance may arise through telomere integrity, NF- κ B signalling, metabolic transcription, or some combination, and this is summarised in cancer oriented syntheses that explicitly treat RAP1/TERF2IP as a multifunctional factor in tumour development¹⁶³. Even within telomere biology, RAP1 can exhibit properties that complicate the description of RAP1 as only an adaptor; for example, biochemical studies have shown direct interactions between human RAP1 and telomeric DNA, as well as effects on TRF2 localisation⁶⁵. Therefore, interventions that alter RAP1 abundance or interaction networks may produce anticancer efficacy through signalling/transcription routes, while also carrying liabilities related to inflammation and metabolism that are not predictable from telomere phenotypes alone.

3.8.2 *Extra-telomeric functions of TRF2*

TRF2 remains central to telomere protection, but it also has a growing body of extra-telomeric evidence that is mechanistically diverse. Early genome wide mapping and follow up analyses showed that TRF proteins can occupy internal genomic loci enriched in interstitial telomeric sequences (ITSs) and satellite DNA, indicating that TTAGGG related binding is not confined to chromosome termini¹⁶⁴. This is relevant because ITSs can occur near genes and within introns, giving TRF2 a route to influence local chromatin structure or transcription factor recruitment. Beyond ITS binding, TRF2 has been linked to G-quadruplex (G4) recognition and broader genome scanning: Mukherjee et al. reported extensive TRF2 binding at extra-telomeric G4 associated loci on a genome wide scale, supporting a model in which TRF2 can function as a sequence/structure sensitive chromatin factor beyond telomeres¹⁶⁵. The transcriptional consequences of this type of occupancy have been worked out in specific promoter systems. A prominent example links TRF2 to angiogenesis: El Maï and colleagues showed TRF2 binding and transactivation at the platelet derived growth factor receptor β (PDGFR β) promoter, connecting TRF2 abundance to neoangiogenic potential as an extratelomeric transcriptional regulator¹⁶⁶. Another gene regulatory axis involves heparan sulfate editing and pro-angiogenic signalling: Zizza et al. reported that TRF2 positively regulates sulfatase 2 (SULF2) and modulates vascular endothelial growth factor A (VEGF-A) release, again supporting a model in which TRF2 controls tumour supporting pathways through transcriptional regulation rather than telomere length control alone¹⁶⁷. TRF2 has also been placed upstream of telomerase gene regulation in a promoter structure framework: Sharma et al. described direct regulation of human TERT via non-telomeric TRF2 interaction with promoter G-quadruplex structures, providing a mechanistic bridge between TRF2 chromatin binding and telomerase reactivation in cancer cells¹⁶⁸. In the same general category of promoter control, TRF2 dependent repression/activation has been shown at the p21 locus through G4 mediated recruitment of chromatin regulators, placing TRF2 in a pathway that intersects with cell cycle control¹⁶⁹. Promoter specificity is also being expanded: Mukherjee et al. reported that TRF2 regulates interleukin-1 receptor type 1 (IL1R1) expression in cancer, linking TRF2 to control of the inflammatory receptor in a telomere length dependent transcriptional network¹⁷⁰. A further example of G4 linked promoter action is TRF2 dependent regulation of the PCGF3 promoter via G-quadruplex motifs, reinforcing the notion that TRF2 can couple DNA secondary structure to transcriptional output¹⁷¹. These studies show that TRF2 can behave as a chromatin bound regulator at selected genes, with outputs that map to angiogenesis, inflammation, telomerase expression, and cell cycle arrest pathways.

Extra-telomeric TRF2 biology also intersects tumour immunology and the DNA damage response. In immune surveillance models, Biroccio et al. showed that TRF2 can limit natural killer cell recruitment and modulate the tumour microenvironment, indicating that TRF2 levels can influence

immune visibility through non-telomeric mechanisms¹⁷². Cherfilis Vicini and colleagues extended this concept by showing that cancer cells can induce immune escape via glycocalyx changes under TRF2 control, emphasising a pathway in which TRF2 affects how tumours interact with immune effector cells¹⁷³. On the genome stability side, TRF2 has been shown to respond to non-telomeric DNA breaks and influence repair pathway choice. Mao et al. provided evidence that TRF2 contributes to the repair of non-telomeric double strand breaks, linking TRF2 to homologous recombination and genome maintenance beyond telomeres¹⁷⁴. TRF2 recruitment kinetics and pathway effects at damage sites have been further dissected using imaging and molecular assays, including detailed analyses of TRF2 dynamics at laser induced lesions and under oxidative stress conditions¹⁷⁵. Extra-telomeric TRF2 roles also extend into replication programmes at heterochromatin: Méndez Bermúdez et al. reported genome wide control of heterochromatin replication by TRF2, supporting a model where TRF2 contributes to replication timing and stability outside telomeric ends¹⁷⁶. In cancer, telomere length itself can reshape TRF2 distribution and transcriptional outputs: Mukherjee et al. reported telomere length dependent TRF2 occupancy and transcriptional regulation in cancer contexts¹⁷⁷, and more recent studies have proposed that telomeres can regulate TERT expression through long range control involving TRF2 dependent mechanisms¹⁷⁸. These results show the possible benefits and risks for TRF2 directed therapeutics: efficacy might come from disrupting telomere protection in highly TRF2 dependent tumours, but toxicity could arise from impairing TRF2 driven angiogenic programmes, immune modulation, or genome wide replication/repair functions that support normal tissue homeostasis.

Chapter 4

Materials and Methods

This chapter describes the materials and methods used in the present study and relates directly to the experiments presented in the Results and Discussion section. It includes the details of the reagents, cell lines, instruments, and experimental procedures used throughout the study, along with the analytical and statistical methods applied for data analysis and interpretation. The purpose of this chapter is to give a clear and organised description of how the study was carried out, so that the experimental workflow can be followed easily and understood in the context of the results obtained. Unless otherwise stated, all experiments were carried out using the respective IC₅₀ concentrations of the tested compounds, as described in the appendix. For convenience, the catalogue numbers or case numbers of the products, reagents, and consumables used in this study are provided separately in the appendix section of the thesis.

4.1 Selection and synthesis of compounds

The original hit compound, A822, which served as the basis for the derivatives investigated in this thesis, was previously identified within the research group as part of the STATEGMED project (3/306853/9/NCBR/2017). The compound was included in an international patent application (WO2023182897A1). The lead compounds investigated in this thesis were obtained following a virtual screening workflow used to identify potential candidates modulating TRF1/TRF2-TIN2 interactions for further biological evaluation. The design and chemical synthesis of these compounds were carried out in the Organic Chemistry Laboratory of Dr. Sławomir Makowiec at Gdańsk University of Technology, Poland, by Ms. Anna Hromova, Mr. Radosław Krzemieniecki, and co-workers. As the chemical synthesis and compound development form part of a separate research project, the detailed synthetic procedures are not included in this thesis. In addition, the chemical structures and associated intellectual property are protected under patent application WIPO ST 10/C PL453805. Therefore, only the biological evaluation and related experimental studies performed using these compounds are described and discussed in the present study. The chemical structures of the compounds are provided in the Appendix.

4.2 Cell culturing and maintenance

A total of 24 cell lines were used in this study, namely A549, HCT116, HeLa, MCF7, HepG2, U-2 OS, A2780, U-87 MG, HEK-293, HaCaT, MRC-5, BT-474, HT29-MTX, MDA-MB-231, SKBR3, T-47D, HT29, LoVo, DU145, CCD 841 CoN, BT-20, MCF7 Tam1, LNCaP, and HMEC. All adherent cell lines were maintained at 37 °C in a humidified incubator under the appropriate carbon dioxide (CO₂) concentration required for each cell type. Unless otherwise specified, the culture media were supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics consisting of penicillin (62.6 µg/mL) and streptomycin (40 µg/mL). Cell cultures were handled under aseptic conditions and were routinely passaged according to their growth characteristics and confluency. Among the cell lines used, MCF7, A549, HaCaT, A2780, BT-20, and LNCaP were cultured in RPMI medium under 5% CO₂. HCT116, U-2 OS, HT29-MTX, and HT29 were maintained in McCoy's medium under 5% CO₂. HepG2, U-87 MG, and HeLa cells were grown in MEM under 5% CO₂. HEK-293, MDA-MB-231, SKBR3, and T-47D were maintained in DMEM under 10% CO₂, whereas MRC-5, DU145, and CCD 841 CoN were cultured in EMEM under 5% CO₂. LoVo cells were maintained in F12K medium under 5% CO₂. The tamoxifen resistant MCF7 Tam1 cells were cultured

in RPMI medium supplemented with 1 μ M 4-hydroxytamoxifen and 10 μ g/mL human insulin. For the remaining cell lines, BT-474 cells were maintained in Hybri-Care medium supplemented with 10% FBS and cultured at 37 °C in 5% CO₂, in accordance with ATCC recommendations. HMEC cells were cultured in Complete Mammary Epithelial Cell Basal Medium under 5% CO₂. All cell lines were routinely examined under an inverted microscope to monitor cell morphology, adherence, confluency, and general growth behaviour. In addition, cultures were regularly screened for mycoplasma contamination and observed for any visible morphological abnormalities. Only healthy, contamination free cultures were used for subsequent experiments. The origin of cell lines and culture conditions are provided in appendix.

4.3 Molecular docking and molecular dynamics simulations

4.3.1 Molecular docking

The crystal structure of the TRF1-TRFH domain in complex with the TIN2 peptide was used as the starting point for the molecular docking studies. The structure was obtained from the Protein Data Bank under the accession code 3BQO, with a reported resolution of 2.0 Å. This structure was selected because it represents the experimentally resolved binding interface between TRF1 and TIN2, which was the region of interest in the present study. The protein structure, after removing TIN2 peptide, was prepared using the Protein Preparation Wizard in the Schrödinger Suite (Maestro v12.8)¹⁷⁹. During this step, crystallographic water molecules were removed, hydrogen atoms were added, and missing side chains and loops were rebuilt using Prime. The prepared structure was then subjected to restrained energy minimisation with the Optimised Potentials for Liquid Simulations (OPLS) 2005 force field.

The docking site was defined based on the TIN2 binding region present in the crystal structure. All ligands included in the study were prepared using LigPrep to generate the most probable protonation and tautomeric states at pH 7.0 $\pm$ 0.5. The resulting ligand structures were further minimised using the OPLS-2005 force field before docking. Molecular docking was performed using the GLIDE module in extra-precision (XP) mode. To allow limited steric flexibility during docking, the van der Waals radii of non-polar ligand atoms were scaled by 0.8, with a partial charge cut-off of 0.15. Default grid settings centred on the co-crystallised binding region were used. For each ligand, the ten highest ranked poses were retained for further evaluation. The docking poses and their intermolecular interactions were visually inspected in PyMOL v3.1¹⁸⁰, and the XP docking score was used as the main criterion for ranking.

4.3.2 Molecular dynamics simulations and trajectory analysis

The top ranked pose among the 10 docking poses generated for each ligand was selected to form the protein ligand complex, which was then subjected to molecular dynamics (MD) simulations to examine their stability and behaviour over time in a solvated environment. MD simulations were carried out using the Desmond simulation package under periodic boundary conditions. Each complex was placed in an orthorhombic simulation box and solvated with the TIP3P water model, with a 10 Å buffer maintained between the solute and the box edges. Counterions were added where required to neutralise the system, and additional Na⁺ and Cl⁻ ions were introduced to achieve a physiological salt concentration of 150 mM. All simulations were performed using the OPLS-2005 force field. Long range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method, while short range Coulombic and van der Waals interactions were calculated with a 9.0 Å cut-off. The equations of motion were integrated using the RESPA multiple time step algorithm, with a 2.0 fs time step for bonded and short range non-bonded interactions and 6.0 fs for long range electrostatic interactions. Bonds involving hydrogen atoms were constrained to ensure stable integration during the simulation. Before the production run, each system underwent a standard relaxation and equilibration procedure. This included initial energy minimisation, followed by short restrained simulations under NVT and NPT (1 ns each) conditions. During the early stages of

equilibration, positional restraints were applied to the heavy atoms of the solute to allow the solvent to equilibrate properly around the complex. The systems were gradually heated to 300 K, and the restraints were then progressively released. After equilibration, an unrestrained 1000 ns production simulation was performed for each selected complex in the NPT ensemble at 300 K and 1.01325 bar. Temperature was maintained using a Langevin thermostat, and pressure was controlled using the Martyna Tobias Klein (MTK) barostat. The total MD simulation time was 1000 ns with a Trajectory frame interval of 0.2 ns (200 ps). To this end, for each simulation, a total of 5000 trajectory frames were recorded and used for further analysis.

The simulation trajectories were analysed using the Simulation Interaction Diagram and related analysis tools available in Desmond, installed on the CI TASK Tryton Plus supercomputer cluster at Gdańsk University of Technology. To assess structural stability during the simulation, the root mean square deviation (RMSD) was calculated, while local flexibility was examined through root mean square fluctuation (RMSF) analysis of the C α atoms. In addition, hydrogen bonds and other protein ligand interactions were monitored throughout the trajectory to evaluate the persistence of key contacts and to determine whether the docked complexes remained stable over time. To further explore the dynamic behaviour of the protein and the influence of ligand binding on residue communication, dynamic cross correlation matrix (DCCM) analysis was performed on the MD trajectories. This analysis was used to identify correlated and anti-correlated motions between residues during the course of the simulation and to examine whether ligand binding altered the internal dynamic network of the TRF1-TRFH domain. In this way, the DCCM analysis provided additional mechanistic insight beyond the static docking poses. Finally, the binding free energy of the selected complexes was estimated using the Prime molecular mechanics/generalized Born surface area (MM/GBSA) method implemented in Schrödinger¹⁸¹. For this purpose, 100 representative frames were extracted from the 1000 ns simulation trajectories at regular intervals, and the average binding free energy was calculated across all selected frames. This approach allowed the estimation of ligand binding affinity while taking into account the conformational changes sampled during the MD simulation.

4.4 Cell cytotoxic evaluation

The cytotoxic activity of 11 synthesised compounds, the native compound A822, and the two reference standard drugs 4-hydroxytamoxifen (4OHT) and etoposide (ETP) was evaluated in a panel of 24 cell lines, namely A549, HCT116, HeLa, MCF7, HepG2, U-2 OS, A2780, U-87 MG, HEK-293, HaCaT, MRC-5, BT-474, HT29-MTX, MDA-MB-231, SKBR3, T-47D, HT29, LoVo, DU145, CCD 841 CoN, BT-20, MCF7 Tam1, LNCaP, and HMEC. Cell viability was determined by the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide obtained from Sigma-Aldrich. Cells were seeded into 96-well culture plates and allowed to attach by overnight incubation under standard culture conditions. After attachment, the cells were treated with different concentrations of the test compounds for 72 h. The concentration range used for the assay was 100 μ M to 0.097 μ M. 1% dimethyl sulfoxide (DMSO) obtained from Merck was used as the solvent control. Following treatment, MTT solution was added to each well at a final concentration of 0.4 mg/mL, and the plates were incubated at 37 °C for 2-3 h to allow formazan crystal formation. After incubation, the medium was removed carefully, and 100 μ L of DMSO was added to each well to dissolve the formed crystals¹⁸². The absorbance was then measured at 450 nm using an ASYS UVM340 microplate reader. The half maximal inhibitory concentration (IC₅₀) value of each compound was calculated from the dose response curves generated by plotting relative cell survival against compound concentration using GraphPad Prism 10. R software version 4.5.2 and the ggplot2 package¹⁸³ were used to generate the IC₅₀ heatmaps. The final results were obtained from the mean of three independent experiments, and the average values were used for further analysis. The selectivity index (SI) was calculated for each compound as:

$$SI = IC_{50} \text{ (non-cancer cell line)} / IC_{50} \text{ (cancer cell line)}$$

IC₅₀ values from different non-cancer cell lines were not averaged, as their sensitivities to the same compound may differ. Therefore, the experimentally determined IC₅₀ of the same compound in the selected non-cancer reference cell line was used for each SI calculation.

4.5 Live cell imaging, growth area analysis, cell tracking, and trypan blue assay

For live cell imaging experiments, HeLa and MCF7 cells were seeded in 48-well plates at densities of approximately 8.9×10^3 cells/cm² and 9.4×10^3 cells/cm², respectively. After seeding, the cells were allowed to attach overnight under standard culture conditions. On the following day, the cells were treated with TRFiN41, TRFiN49, TRFiN72, and 4OHT at the respective IC₅₀ concentrations indicated in appendix. Immediately after treatment, the plates were transferred to an Olympus IX83 inverted wide field microscope fitted with a cellVivo-3 Variant 1 incubator system to maintain appropriate culture conditions during imaging. Time lapse images were acquired at 1 h intervals for a total duration of 72 h. The acquired image sequences were analysed using Olympus cellSens Dimension software, and the cellular growth area at each time point was measured. The obtained data were used to generate growth curves in GraphPad Prism 10¹⁸⁴. Cell movement in two dimensional culture conditions was analysed from the same time lapse image sets using CellTraxx v4.6¹⁸⁵. The trajectory data obtained from CellTraxx were subsequently replotted using the ggplot2 package in R software version 4.5.2 to obtain clear visualisation of cell movement patterns. The effect of compound treatment on cell death was further examined at 24, 72, 120, and 168 h using the trypan blue exclusion method. At each selected time point, both live and dead cells were counted with a Corning cell counter, and the numbers were used to assess the extent of treatment associated cell death over time.

4.6 Surface plasmon resonance analysis

Surface plasmon resonance analysis was performed using a Biacore T200 system (Cytiva) according to the manufacturer's instructions¹⁸⁶. The experiment was performed at the Intercollegiate Faculty of Biotechnology of the University of Gdańsk, Poland. The immobilisation of TRF1 protein was carried out below the isoelectric point of the protein to obtain a positively charged protein surface for coupling. Buffers within the pH range of 3.5-5.5 were first tested for immobilisation, and 10 mM sodium acetate buffer at pH 4.5 was selected as the suitable immobilisation buffer. TRF1 protein was immobilised on a CM4 Sensor Chip (Cytiva) using 10 mM sodium acetate buffer at pH 4.5. The immobilisation level of TRF1 protein was around 10000 response units (RU). The interaction between TRF1 protein and TIN2 peptide was first analysed to confirm that the functional region of immobilised TRF1 was active and properly folded. A positive interaction between TRF1 and TIN2 peptide was used as confirmation for further binding analysis. Binding analysis of TRFiN compounds and A822 with TRF1 protein was carried out using the TRF1 immobilised CM4 Sensor Chip. Serial dilutions of the analytes were prepared in PBS-P buffer (Cytiva) and injected over the prepared sensor chip surface. PBS-P buffer was used as the running buffer in all experiments. All surface plasmon resonance analyses were performed at 25 °C. For kinetic experiments, the buffer flow rate was maintained at 30 µL/min. After each analysis, the sensor chip surface was regenerated using 1.5 M NaCl and 10 mM glycine buffer at pH 3.0. An additional wash with 50% DMSO was performed after each analysis to remove remaining analyte from the chip surface. The obtained data were analysed using Biacore T200 Evaluation Software (Cytiva). Sensorgrams were prepared after subtraction of the background response from the reference flow cell and the control response obtained from buffer injection. At least three kinetic experiments were performed for each analyte.

4.7 Sample preparation and acquisition for flow cytometry analysis

For all flow cytometry experiments, HeLa and MCF7 cells were seeded in complete culture medium on suitable culture plates. For 24 h and 72 h treatment experiments, cells were seeded at a density of 1.43×10^4 cells/cm². For 120 h and 168 h treatment experiments, cells were seeded at a density of 9.5×10^3 cells/cm². The cells were maintained under standard culture conditions and allowed to attach overnight. After overnight adhesion, the cells were treated with the experimental compounds

at their respective IC_{50} for the required duration of each experiment. After the completion of treatment and staining, the samples were acquired using an Agilent NovoCyte flow cytometer. For each sample, 10,000 events were recorded. Fluorescence signals were collected using the appropriate channels based on the staining method. The fluorescein isothiocyanate (FITC) channel was used for green fluorescence signals. The PI channel was used for propidium iodide staining, and the PerCP channel was used for 7-AAD staining. The acquired flow cytometry data were analysed using FlowJo v10 software. Each experiment was performed independently three times¹⁸⁷.

4.8 Cell cycle analysis by BrdU and propidium iodide staining

Cell cycle analysis was performed using combined 5-bromo-2'-deoxyuridine (BrdU) and PI staining¹⁸⁸. This method was used to analyse DNA content and active DNA synthesis in the same cell population. Propidium iodide was used to measure total DNA content, while BrdU incorporation was used to identify cells undergoing DNA synthesis. HeLa and MCF7 cells were seeded in complete culture medium on suitable culture plates. For 24 h and 72 h treatment experiments, cells were seeded at a density of 1.43×10^4 cells/cm². For 120 h and 168 h treatment experiments, cells were seeded at a density of 9.5×10^3 cells/cm². Cells were maintained under standard culture conditions and allowed to attach overnight. After overnight adhesion, cells were treated with the experimental compounds at their respective IC_{50} concentrations for the required time points. Twelve hours before the end of each treatment period, BrdU (Sigma-Aldrich) was added to the culture medium at a final concentration of 20 μ M and cells were further incubated at 37 °C. After treatment, cells were collected by trypsinisation and centrifuged at 1030 rpm. The cell pellet was washed once with 1X phosphate buffered saline (PBS) and centrifuged again under the same conditions. The supernatant was discarded and the cells were fixed by adding 2 mL of ice cold 70% ethanol. The samples were kept on ice for 1 h or stored at -20 °C until further staining. Before staining, fixed cells were rehydrated in 2 mL of 1X PBS for 15 min at room temperature and centrifuged. The cell pellet was resuspended in 1 mL of freshly prepared 2 M hydrochloric acid in 1X PBS and incubated for 30 min at room temperature to denature the DNA. After centrifugation, the pellet was resuspended in 2 mL of 0.1 M sodium tetraborate buffer at pH 8.5 and incubated for 10 min at room temperature for neutralisation. The samples were then centrifuged and resuspended in 1 mL of 0.1% Triton X-100 in 1X PBS for 15 min at room temperature. After incubation, 2 mL of 1X PBS was added and the samples were centrifuged. For blocking, the cell pellet was resuspended in blocking buffer containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in 1X PBS. The samples were incubated for 15 min at room temperature and centrifuged. The pellet was then resuspended in 150 μ L of Alexa Fluor® 488-conjugated anti-BrdU antibody (BU1/75 (ICR1)) prepared at 1:150 dilution. The samples were incubated for 30 min at 37 °C or overnight at 4 °C. After antibody staining, 1 mL of PI staining solution containing 100 μ g/mL RNase A and 23 μ g/mL propidium iodide in 1X PBS was added to each sample. The samples were incubated for 30 min to 1 h at room temperature. After incubation, cells were washed by adding 3 mL of 1X PBS and centrifuged. The final pellet was resuspended in 400 μ L of 1X PBS and acquired using an Agilent NovoCyte flow cytometer. BrdU and PI signals were detected using the FITC and PI channels, respectively. The cell populations were quantified from the BrdU/PI dot plots. Histograms were used only for visualisation of DNA content distribution. The quantified data were exported to GraphPad Prism 10 for statistical analysis.

4.9 DNA fibre assay for analysis of replication fork progression

DNA fibre assay was performed to analyse replication fork progression in HeLa and MCF7 cells after treatment with the experimental compounds¹⁸⁹. Cells were seeded in complete culture medium on suitable culture plates at a density of 9.5×10^3 cells/cm² for 168 h treatment experiments. The cells were maintained under standard culture conditions and allowed to attach overnight. After overnight adhesion, the cells were treated with the experimental compounds at their IC_{50} for the required treatment period. After treatment, the culture medium was removed and the cells were washed once with sterile 1X PBS. Fresh complete medium containing the same compound concentration and 50 μ M

5-iodo-2'-deoxyuridine (IdU; Cayman Chemical) was added to the cells. The plates were incubated at 37 °C for 30 min to label newly synthesised DNA with IdU in the presence of the compound. The medium containing IdU was then removed and the cells were gently washed three times with sterile 1X PBS. Fresh complete medium containing the same compound concentration and 150 µM 5-chloro-2'-deoxyuridine (CldU; Cayman Chemical) was added to the cells and incubated again at 37 °C for 30 min. After CldU labelling, the medium was removed and the cells were washed three times with sterile 1X PBS. The cells were detached using 0.25% trypsin ethylenediaminetetraacetic acid (trypsin-EDTA) for 2-5 min, depending on cell detachment from the plate surface. The cell suspension was collected and centrifuged at 1030 rpm for 5 min at 4 °C. The supernatant was removed carefully and the cell pellet was resuspended in 4 mL of 1X PBS. A 2.5 µL drop of the cell suspension was placed on a positively charged microscopic slide near the frosted end. Two to three drops were placed on each slide. To each drop, 7.5 µL of lysis buffer containing 200 mM Tris-HCl pH 7.5, 50 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS) was added. The drop was mixed gently by pipetting 4-5 times and incubated for 7-8 min at room temperature to lyse the cells and release the DNA.

After lysis, the slide was tilted at a 45° angle to allow the DNA containing drop to run down the slide and form stretched DNA fibres. The slides were air dried for 15-20 min until a visible white line was formed. The DNA fibres were fixed by placing the slides in a Coplin jar containing freshly prepared ice cold methanol:acetic acid at a 3:1 ratio for 5 min. After fixation, the slides were air dried and were either stored at -20 °C or processed directly for immunostaining. For immunostaining, the slides were washed twice with 1X PBS for 5 min each in a Coplin jar. DNA was denatured by treating the slides with 2.5 M hydrochloric acid for 1 h at room temperature. The slides were then washed three times with 1X PBS for 5 min each to remove the acid. Blocking was performed using blocking buffer containing 3% BSA and 0.1% Triton X-100 in 1X PBS for 30-45 min at room temperature. After blocking, the slides were washed twice with 1X PBS before primary antibody incubation. The slides were incubated with 50 µL of primary antibody solution for 2 h 30 min at room temperature in a humid chamber. Incorporated IdU was detected using mouse monoclonal anti-BrdU antibody (Becton Dickinson, 347580) at a dilution of 1:100. Incorporated CldU was detected using rat anti-BrdU antibody (Abcam, ab6326) at a dilution of 1:100. After primary antibody incubation, the slides were washed three times with 1X PBS for 5 min each. The slides were then incubated with 50 µL of secondary antibody solution for 1 h at room temperature in a humid chamber. IdU was detected using Alexa Fluor 488 goat anti-mouse secondary antibody at a dilution of 1:200, and CldU was detected using Alexa Fluor 594 goat anti-rat secondary antibody at a dilution of 1:200. After secondary antibody staining, the slides were dried and mounted with 20 µL of ProLong Gold Antifade Mountant (Invitrogen, P36930). A coverslip was placed carefully over each sample and the slides were allowed to dry at room temperature in the dark. The stained slides were stored at 4 °C for short term storage or at -20 °C for long term storage. DNA fibres were imaged using a ZEISS LSM 800 confocal microscope with Airyscan using a 60x oil immersion objective. The lengths of green IdU labelled fibres and red CldU labelled fibres were measured using ImageJ software. The IdU/CldU ratio was calculated from the measured fibre lengths. The quantified data were exported to GraphPad Prism 10 for statistical analysis.

4.10 Analysis of DNA damage by γH2AX and 7-AAD staining

DNA damage was analysed by flow cytometry using Alexa Fluor 488-conjugated anti-H2AX antibody and 7-AAD staining¹⁹⁰. HeLa and MCF7 cells were seeded in complete culture medium on suitable culture plates. For 24 h and 72 h treatment experiments, cells were seeded at a density of 1.43×10^4 cells/cm². For 120 h and 168 h treatment experiments, cells were seeded at a density of 9.5×10^3 cells/cm². Cells were maintained under standard culture conditions and allowed to attach overnight. After overnight adhesion, the cells were treated with the experimental compounds at their IC₅₀, as provided in Appendix, for 24 h, 72 h, 120 h, and 168 h. After treatment, the cells were collected by trypsinisation. The cell pellet was fixed in 2 mL of ice cold 75% ethanol and stored at -20 °C until further analysis. Before staining, the fixed cells were rehydrated with 2 mL of PBS on ice for 15 min.

The samples were then centrifuged at 1030 rpm for 5 min at 4 °C and the supernatant was removed. The cell pellet was resuspended in 0.5 mL of 0.2% Triton X-100 in 1X PBS and incubated for 15 min at room temperature. After incubation, 2 mL of 1X PBS was added and the samples were centrifuged again. The cell pellet was resuspended in 1 mL of blocking buffer containing 3% BSA and 0.1% Triton X-100 in 1X PBS. The samples were incubated for 1 h at room temperature. After blocking, 2 mL of 1X PBS was added and the samples were centrifuged. The pellet was then resuspended in 200 µL of Alexa Fluor 488-conjugated anti-H2AX antibody solution (BD Biosciences) prepared at 1:200 dilution. The samples were incubated for 1 h at 37 °C or overnight at 4 °C. After antibody staining, 200 µL of staining solution containing 100 µg/mL RNase A and 3.3 µM 7-AAD was added to each sample. The samples were incubated for 30 min at room temperature. After incubation, 2 mL of 1X PBS was added and the cells were centrifuged. The final cell pellet was resuspended in 400 µL of 1X PBS and acquired using an Agilent NovoCyte flow cytometer. Alexa Fluor 488 and 7-AAD signals were detected using the suitable fluorescence channels. The flow cytometry data were analysed using FlowJo v10 software. The quantified data were exported to GraphPad Prism 10 for statistical analysis.

4.11 Assessment of mitochondrial transmembrane potential by JC-1 staining

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was analysed using JC-1 staining¹⁹¹. JC-1 dye accumulates in mitochondria depending on the mitochondrial membrane potential. In cells with intact mitochondrial membrane potential, JC-1 forms aggregates and gives red fluorescence. In cells with reduced mitochondrial membrane potential, JC-1 remains in monomeric form and gives green fluorescence. The change in mitochondrial membrane potential was analysed by measuring the JC-1 aggregate to monomer fluorescence ratio using flow cytometry. Cells were seeded in complete culture medium and treated with the experimental compounds at the required concentrations and time points as described in the previous sections. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma-Aldrich) was used as a positive control for mitochondrial depolarisation. For FCCP treated samples, cells were treated with 10 µM FCCP for 30 min before the end of the incubation period. After treatment, the cells were collected by trypsinisation using trypsin-EDTA. The cell suspension was centrifuged at 1030 rpm for 5 min at 4 °C. The supernatant was removed and the cell pellet was resuspended in warm fresh complete medium containing 5 µg/mL JC-1 dye (Sigma-Aldrich). The cells were incubated for 20 min at 37 °C in the dark. After JC-1 staining, the cells were centrifuged and the pellet was washed with 2 mL of 1X PBS. The samples were centrifuged again and the final cell pellet was resuspended in 400 µL of 1X PBS. The fluorescence signal was acquired using an Agilent NovoCyte flow cytometer using the FITC and PI channels. The JC-1 aggregate to monomer ratio was calculated from the acquired fluorescence values. The quantified data were exported to GraphPad Prism 10 for statistical analysis.

4.12 Transmission electron microscopy (TEM) analysis

TEM was performed to examine the ultrastructural changes in cells after treatment with TRFiN compounds. After the required treatment period, the culture medium was removed and the cells were washed with 1X PBS. The cells were detached using trypsin and collected by centrifugation at 1030 rpm for 5 min. The cell pellet was fixed in 1 mL of 2.5% glutaraldehyde solution (Agar Scientific). After primary fixation, the samples were post-fixed using a solution containing 2% osmium tetroxide and 0.1% potassium ferricyanide. Both reagents were obtained from Agar Scientific. After post-fixation, the samples were dehydrated using a graded ethanol series from 30% to 100%. The dehydrated samples were embedded in EPON resin (Agar Scientific). After polymerisation of the resin, ultrathin sections of 65 nm thickness were prepared using a Leica UC7 ultramicrotome. The sections were stained with Uranyless and Reynolds lead citrate (Delta Microscopies). The stained sections were examined using a Tecnai Spirit BioTWIN transmission electron microscope operated at 120 kV.

4.13 Analysis of apoptosis and caspase-3/7 activation by flow cytometry

Apoptosis and caspase-3/7 activation were analysed by flow cytometry after treatment of HeLa cells with TRFiN compounds. Cells were seeded and treated with the experimental compounds as described in the previous sections. After the required treatment period, the culture medium was removed and the cells were collected using phosphate buffered saline ethylenediaminetetraacetic acid (PBS-EDTA). The collected cells were divided into two separate tubes for Annexin V staining and caspase-3/7 activation assay. The cells were centrifuged at 1030 rpm for 5 min at 4 °C. The supernatant was removed and the cell pellet was washed once with 1X PBS. The samples were centrifuged again under the same conditions and used for staining. For apoptosis analysis, cells were stained using FITC-Annexin V apoptosis detection kit (Thermo Fisher Scientific, V13242) according to the manufacturer's instructions with slight modifications. The cell pellet was resuspended in 100 µL of 1X Annexin binding buffer. To each sample, 0.25 µL of FITC-Annexin V and 0.25 µL of 7-aminoactinomycin D (7-AAD; 5 mM stock) were added. The samples were mixed gently and incubated for 15 min on ice. After incubation, 300 µL of 1X Annexin binding buffer was added to each sample before acquisition.

For caspase activation analysis, cells were stained using CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, C10427) according to the manufacturer's instructions with slight modifications. The cell pellet was resuspended in 100 µL of 1X PBS. To each sample, 0.25 µL of CellEvent™ Caspase-3/7 Green detection reagent and 0.25 µL of 7-AAD were added. The samples were incubated for 30 min at 37 °C in the dark. After incubation, 300 µL of 1X PBS was added to each sample before acquisition. The fluorescence signal was acquired using an Agilent NovoCyte flow cytometer. FITC and PerCP channels were used to detect Annexin V or CellEvent™ Caspase-3/7 Green signal and 7-AAD signal, respectively. The acquired data were used for the analysis of apoptotic cell population and caspase-3/7 positive cells¹⁹².

4.14 General immunofluorescence staining procedure

All immunofluorescence staining experiments used in this thesis were performed using a common staining procedure unless stated otherwise. HeLa and MCF7 cells were seeded in complete culture medium on sterile coverslips placed in suitable cell culture plates. For 24 h and 72 h treatment experiments, cells were seeded at a density of 1.43×10^4 cells/cm². For 120 h and 168 h treatment experiments, cells were seeded at a density of 9.5×10^3 cells/cm². The cells were maintained under standard culture conditions and allowed to attach overnight. After overnight adhesion, the cells were treated with the experimental compounds at their IC₅₀ for the required time points. After treatment, the culture medium was removed and the cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. The coverslips were washed with PBST containing 0.2% Triton X-100 in 1X PBS for 10 min, followed by two washes with 1X PBS. The cells were then blocked with blocking buffer containing 2.5% BSA and 0.1% Triton X-100 in 1X PBS for 1 h at room temperature in a humid chamber. After blocking, the coverslips were incubated with the required primary antibody diluted in blocking buffer and kept overnight at 4 °C. The antibodies and dilutions used in this dissertation are provided in the appendix. After primary antibody incubation, the coverslips were washed once with 1X PBS. The cells were then incubated with the respective secondary antibody diluted in blocking buffer for 1 h 30 min at 37 °C in a humid chamber. After secondary antibody incubation, the coverslips were washed twice with 1X PBS for 3 min each. When nuclear staining was required, the coverslips were incubated with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at a concentration of 1 µg/mL in PBST for 10 min at room temperature in the dark. The coverslips were then washed twice with 1X PBS for 3 min each. The stained coverslips were mounted using ProLong Gold Antifade Mountant (Invitrogen) and allowed to dry before imaging¹⁹³.

For live cell nuclear staining, cells were incubated with Hoechst 33342 (Sigma-Aldrich) at a concentration of 0.5 µg/mL for 10 min. For mitochondrial staining, cells were incubated with

MitoTracker Green (Thermo Fisher Scientific) at a concentration of 0.5 μM for 30 min. For CellTracker Green staining, cells were incubated with CellTracker Green (Invitrogen) at a concentration of 50 μM in the respective serum free culture medium for 30 min. After incubation, the cells were centrifuged and the staining medium was replaced with the respective complete culture medium. For 7-AAD staining, cells were incubated with 7.25 μM 7-AAD for 15 min. For DAPI staining, cells were incubated with 1 $\mu\text{g/mL}$ DAPI.

4.15 Cellular senescence analysis by flow cytometry and β galactosidase staining

Cellular senescence was analysed in HeLa and MCF7 cells after treatment with TRFiN compounds. Cells were seeded and treated with the experimental compounds as described in the previous sections. Senescence positive cells were quantified by flow cytometry using the CellEvent™ Senescence Green Detection Kit (Invitrogen) according to the manufacturer's protocol with slight modifications¹⁹⁴. After the required incubation period, the cells were collected by trypsinisation and centrifuged at 1000 rpm for 5 min at 4 °C. The supernatant was removed and the cell pellet was washed once with 1X PBS. The cells were fixed with 2% PFA for 10 min at room temperature. After fixation, 2 mL of 1X PBS was added and the samples were centrifuged. The cell pellet was then resuspended in 1 mL of 1% BSA in PBS to remove the remaining fixation solution and centrifuged again. The staining working solution was prepared by warming the CellEvent™ Senescence Buffer to 37 °C and diluting the CellEvent™ Senescence Green Probe into the pre-warmed buffer at the required working concentration. The cell pellet was resuspended in 100 μL of the pre-warmed working solution. The samples were covered with plastic film to prevent moisture loss and incubated for 2 h at 37 °C without CO_2 in the dark. Incubation was carried out without CO_2 because CO_2 changes the pH of the reaction buffer. After incubation, 2 mL of 1X PBS was added and the samples were centrifuged. The final cell pellet was resuspended in 400 μL of 1X PBS and acquired using an Agilent NovoCyt flow cytometer through the FITC channel.

For microscopic analysis of senescence associated β galactosidase activity, cells were seeded on sterile coverslips at the cell densities and treatment time points described in the previous sections. After treatment, the cells were washed twice with 1X PBS and fixed with a solution containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature. After fixation, the cells were washed twice with 1X PBS. The fixed cells were incubated with β galactosidase staining buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 150 mM sodium chloride, and 1 mg/mL X-Gal in 40 mM citrate/sodium phosphate buffer at pH 6.0. The coverslips were incubated for 16 h at 37 °C without CO_2 . After staining, the cells were washed with 40 mM citric acid/phosphate buffer at pH 4.0, followed by two washes with 1X PBS. The coverslips were mounted using mounting medium containing 90% glycerol and 2.5 mg DABCO. Images were acquired using an Olympus IX83 inverted motorised fluorescence microscope system. The flow cytometry data were exported for statistical analysis¹⁹⁵.

4.16 Colony formation assay

Colony formation assay was performed to analyse the long term survival of HeLa and MCF7 cells after treatment with TRFiN compounds¹⁹⁶. HeLa cells were seeded at a density of 1000 cells per well and MCF7 cells were seeded at a density of 1200 cells per well in 6-well plates. Cells were seeded directly onto the plate surface in complete culture medium and allowed to attach for 24 h under standard culture conditions. After attachment, the cells were treated with TRFiN compounds at concentrations of 1 μM , 3 μM , 6 μM , 12 μM , and 20 μM . The standard compound 4OHT was also used at the same concentration range. After compound exposure, the treatment medium was removed and replaced with fresh complete culture medium. The cells were then maintained under standard culture conditions for eight days to allow colony formation. After incubation, the culture medium was removed and the wells were washed with 1X PBS to remove residual medium and compounds. The colonies were fixed first with 4% PFA for 20 min, followed by fixation with 99.5% methanol. The fixed

colonies were stained with 0.5% crystal violet solution for 20 min at room temperature. After staining, the plates were washed gently and air dried. Images of the stained plates were acquired using an Azure 300 imaging system. Colony formation was quantified by measuring the crystal violet stained colony area using ImageJ software. The values obtained from treated samples were compared with untreated control samples.

4.17 Three dimensional spheroid formation and fluorescence imaging

Three dimensional spheroid formation and growth experiments were performed using agar moulds prepared with the 3D Petri Dish® system (Microtissues®)¹⁹⁷. Agar was prepared in normal saline, liquefied, sterilised, and cast into the 3D Petri Dish® mould. After solidification, the agar moulds were gently removed, rinsed with the respective complete culture medium, and placed in 24-well plates. The required number of cells ($\sim 1.13 \times 10^5$ for HeLa and MCF7) was added to each agar mould in complete culture medium and allowed to settle for 20 min under standard culture conditions. After this initial settling period, additional complete medium containing the required drug dilutions was added carefully to each well without disturbing the cells in the mould. The spheroids were maintained under standard culture conditions for the required experimental period. Live cell imaging of spheroid growth was performed using an Olympus CellVivo inverted microscope. The length of each spheroid structure was measured using Olympus CellSens software.

For fluorescence imaging, spheroids were stained after the final time point. To each spheroid, 0.25 μL of FITC-Annexin V, 0.25 μL of 7-aminoactinomycin D (7-AAD; 5 mM stock), and Hoechst 33342 at a final concentration of 0.5 $\mu\text{g/mL}$ were added. The spheroids were incubated for 10 min at room temperature. After staining, the spheroids were carefully transferred into 1% gelatin solution (Sigma-Aldrich) maintained at a hand tolerable temperature. Each spheroid was carefully aspirated using a 1 mL cut pipette tip and placed on a microscopic slide. The gelatin was allowed to cool and solidify for 3 min. The mounted spheroids were imaged using a ZEISS LSM 800 inverted laser scanning confocal microscope with an Airyscan detector. Imaging was performed using a 63×1.4 NA Plan Apochromat objective. Z stack imaging was performed to allow further analysis of fluorescence distribution within the spheroid structure.

4.18 Wound healing assay for cell migration analysis

The anti-migratory activity of TRFiN compounds was evaluated using a wound healing assay¹⁹⁸. HeLa and MCF7 cells were seeded at a density of 1.59×10^5 cells into ibidi silicone culture inserts placed in glass bottom 24-well plates suitable for live cell imaging. Cells were seeded in the respective complete culture medium and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO₂. After overnight incubation, the silicone inserts were gently removed to create a uniform wound gap. Cellular debris was removed by washing the wells twice with filtered fresh medium. To reduce the contribution of cell proliferation during the assay, cells were pre-treated with 10 $\mu\text{g/mL}$ mitomycin C (Sigma-Aldrich) for 1 h. After mitomycin C treatment, the cells were washed twice with fresh medium. The cells were then treated with TRFiN compounds at concentrations of 2 μM , 5 μM , and 10 μM . Sorafenib was used as a positive control at a concentration of 4.5 μM . The plates were transferred into a CellVivo incubation system (Olympus) maintained at 37 °C with 5% CO₂. Live cell imaging was performed using an IX83 inverted phase contrast fluorescence microscope (Olympus) connected to an XC50 digital colour camera (Olympus). Time lapse images were acquired every 1 h for 72 h at 10X magnification. The acquired images were assembled into time lapse image series using CellSens software (Olympus). Wound gap area was measured using ImageJ software. The average wound width, wound area over time, and cell migration velocity were calculated from the measured wound gap values. Migration velocity was expressed as $\mu\text{m/h}$.

4.19 CAM assay for angiogenesis and tumour growth analysis

Fertilised chicken and quail eggs were obtained from a local supplier (Daniels farm, Ceranow, Poland) and incubated at 37.5 °C with 65% humidity and periodic rotation for three days. On the third day of incubation, the eggs were opened carefully into sterile weighing boats. Each weighing boat was covered with a sterile lid and placed in a bacteriological incubator maintained at 37 °C with 60% humidity. For angiogenesis analysis the chicken embryos were used, sterile glass filter paper discs were prepared with the required concentrations of the experimental compounds and allowed to dry. On the seventh day of incubation, the prepared discs were placed on the vascular region of the chorioallantoic membrane (CAM). Vascular development was monitored after treatment and images were captured at the required time points. The acquired images were analysed using an in house Python script (provided in the flash drive with the thesis) to quantify vascular growth. For the CAM tumour model, quail embryos were used on the fourth day of incubation. A small window was made on the ventral side of the CAM. HeLa or MCF7 cells were prepared in Matrigel (Corning), and 2×10^6 cells in 15 μ L Matrigel were carefully deposited onto the CAM surface. The embryos were incubated for 48 h to allow tumour cell attachment and growth. After incubation, a sterile silicone ring was placed around the inoculation site, and the required drug solution prepared in sterile normal saline was added inside the ring. After treatment of 48 h, the embryos were sacrificed by cutting the vitelline arteries. The tumours were excised carefully from the CAM, weighed, and fixed in 4% PFA. Fixed tumour tissues were processed for cryosectioning using a Leica cryotome as described in the following section. Tissue sections were stained with haematoxylin and eosin (H&E) for histological analysis¹⁹⁹.

The CAM experiments were carried out at a developmental stage that is not classified as animal experimentation under European regulations, including EU Directive 2010/63/EU and European Parliament resolution 2021/2784(RSP). Therefore, separate animal ethical clearance was not required for these experiments. All procedures were performed according to the standard operating procedure provided in the supplementary file. Where applicable, the research was conducted in accordance with the principles of the Declaration of Helsinki.

4.20 Cryosectioning and haematoxylin and eosin staining of CAM tissue and embryonic organs

Cryosectioning was performed for CAM tissue, CAM grown tumours, and chicken embryo organs using the same procedure. The dissected tissue was handled carefully to avoid damage to the soft and highly vascularised embryonic tissue. The collected CAM tissue with tumour or embryonic organ samples were immersed in 4% PFA for 15 min to 1 h at 4 °C. After fixation, the tissues were gently rinsed three times with 1X PBS. For cryoprotection, the fixed tissues were transferred through a sucrose gradient to reduce freezing artefacts. The samples were first incubated in 10% sucrose for 3 h at 4 °C, followed by incubation in 30% sucrose overnight at 4 °C. After cryoprotection, the tissue was placed in a tissue embedding mould containing optimal cutting temperature (OCT) compound (Leica Biosystems). The tissue was positioned so that the required cutting plane was kept perpendicular or diagonal to the cryostat blade. The embedded tissue block was frozen directly on the freezing stage of the cryostat until completely solidified. The frozen blocks were stored at -80 °C until sectioning.

Cryosectioning was performed using a Leica Biosystems cryostat. The cryostat chamber and blade temperature were maintained between -15 °C and -25 °C. Tissue sections were cut at a thickness of 15 μ m and mounted onto charged microscope slides (X tra Slides, Leica Biosystems). The sections were then processed for haematoxylin and eosin (H&E) staining. For H&E staining, the slides were rinsed in 1X PBS for 3 min. The slides were then washed under a gentle stream of tap water for 3 min to remove OCT compound from the sections. The sections were stained with Mayer's haematoxylin (Leica Biosystems) for 30 s and washed under a gentle stream of tap water for 1 min. Nuclear bluing was performed by dipping the slides in 1X PBS for 20 s, followed by washing under tap water for 1 min. The slides were then dipped in 70% ethanol for 30 s and 95% ethanol for 30 s. Counterstaining was performed using Alcoholic eosin (Leica Biosystems) for 30 s. After eosin staining, the sections were dehydrated through two changes of 95% ethanol for 15 s each and three changes of 100% ethanol

for 15 s each. The slides were cleared through three changes of xylene for 1 min each. After clearing, the sections were air dried and mounted using Entellan mounting medium (Sigma-Aldrich). Coverslips were placed carefully over the sections and the slides were allowed to dry. The stained sections were imaged using an Olympus IX83 inverted motorised microscope system.

4.21 Acridine orange staining for analysis of autophagy associated acidic vesicular organelles

Autophagy associated acidic vesicular organelles were analysed by acridine orange staining followed by flow cytometry. HeLa and MCF7 cells were seeded in complete culture medium in 6-well plates at a density of 9.5×10^3 cells/cm². The cells were seeded in triplicate and allowed to attach before treatment. After attachment, the cells were treated with TRFiN41, TRFiN49, TRFiN72, and 4OHT at their respective IC₅₀ values, as described previously. The treatment was continued up to 168 h. Untreated cells were used as the negative control. Cells incubated in Hank's balanced salt solution (HBSS) for 5-6 h were used as a positive control for starvation induced autophagy. At the end of treatment, both adherent and floating cells were collected to avoid the loss of detached or damaged cells. The cells were washed with PBS and stained with acridine orange (3 μ M) under optimised staining conditions. After staining, the cells were washed and resuspended in PBS for flow cytometric acquisition. The stained cells were acquired using a NovoCyte flow cytometer. Acridine orange green fluorescence was collected in the B530-A channel, and red fluorescence was collected in the B675-A channel. Forward scatter and side scatter were used to exclude debris and poorly resolved events before fluorescence analysis. The formation of acidic vesicular organelles was quantified using the acridine orange red to green fluorescence intensity ratio (AO R/GFIR). For each cell, the AO R/GFIR value was calculated by dividing the red acridine orange fluorescence intensity by the green acridine orange fluorescence intensity.

$$\text{AO R/GFIR} = \text{red fluorescence intensity} / \text{green fluorescence intensity}$$

The untreated control population was used to define the red to green fluorescence intensity ratio threshold (R/GFIR-T) separately for each cell line. Events with AO R/GFIR values above the R/GFIR-T were considered AO R/GFIR positive cells. The same R/GFIR-T was applied to treated samples and to the HBSS starved positive control of the corresponding cell line. The percentage of AO R/GFIR positive cells was calculated for each replicate. Final values were expressed as mean $\pm$ standard error of the mean from triplicate samples. This ratiometric analysis was used to measure acidic vesicular organelle accumulation by normalising red acridine orange fluorescence to the total acridine orange staining signal of each cell²⁰⁰.

4.22 Telomere fluorescence *in situ* hybridisation (Telo-FISH)

HeLa and MCF7 cells were seeded in complete culture medium at a density suitable for 168 h treatment, such as 9.5×10^3 cells/cm², and were allowed to attach overnight under standard culture conditions. After attachment, the cells were treated with TRFiN41, TRFiN49, and TRFiN72 at their respective IC₅₀ values. Untreated cells were used as the negative control. The treatment was continued for 168 h, and both adherent and floating cells were collected at the end of treatment to avoid the loss of damaged or detached cells.

4.22.1 Telo-FISH in metaphase spreads

Metaphase fluorescence *in situ* hybridisation (FISH) was performed to examine telomeric signals on metaphase chromosomes after 168 h treatment with TRFiN compounds. At the end of treatment, colcemid was added to the culture medium at a final concentration of 0.3 μ g/mL, and the cells were incubated at 37 °C for 1 hr 30 min-2 hr 30 min to arrest cells in metaphase. After incubation, the culture medium was removed, and 8 mL of pre-warmed Ohnuki buffer was added to each plate. Ohnuki buffer was used for hypotonic swelling of the cells [0.205 g potassium chloride (KCl), 0.1175 g sodium nitrate (NaNO₃), and 0.045 g trisodium citrate dihydrate (C₆H₅Na₃O₇·2H₂O) dissolved in distilled water and made up to 100 mL]. The cells were incubated in Ohnuki buffer for 20 min at 37 °C. After

hypotonic treatment, the cells were harvested using a cell scraper and transferred into 15 mL centrifuge tubes. The cell suspension was centrifuged at 1200 rpm for 10 min at room temperature. The supernatant was removed, and the pellet was gently resuspended while the tube was placed on a vortex. Ice cold fixative was added dropwise to the pellet to a final volume of 3 mL (methanol:acetic acid, 3:1, v/v), and the cells were incubated for 30 min at 2-8 °C. The cells were centrifuged again at 1200 rpm for 10 min, the supernatant was removed, and the pellet was resuspended in fresh cold fixative. The fixation step was repeated once, and the fixed cells were stored overnight at 2-8 °C. For slide preparation, the heating block was set to 40 °C. The spreading was performed under room conditions of approximately 20-25 °C and 40-60% humidity. The fixed cell suspension was dropped onto cold clean glass slides held at a slight angle. The pipette was held approximately 30 cm above the slide to improve chromosome spreading. The slides were briefly breathed on to increase local humidity and improve spreading. The slides were then gently washed with 50% acetic acid (acetic acid:distilled water, 1:1, v/v) and placed on the heating block to dry.

The dried slides were dehydrated through an ethanol series by dipping in 70% ethanol for 3 min, 90% ethanol for 2 min, and 100% ethanol for 2 min. The slides were removed from ethanol and allowed to dry completely. Telomere hybridisation was performed using an Alexa Fluor 647 labelled telomere peptide nucleic acid (PNA) probe at a concentration of 0.3 µg/mL prepared in PNA hybridisation solution (70% deionised formamide, 0.25% blocking reagent (Roche), and 10 mM Tris-HCl, pH 7.5). For each slide, 50 µL of the PNA probe solution was added, and a coverslip was placed carefully without air bubbles. All subsequent steps were performed in the dark. The slides were denatured at 80 °C for 5 min and then transferred to a humidified chamber for overnight hybridisation at room temperature in the dark. After hybridisation, the coverslips were removed, and the slides were washed with PNA wash A for 5 min (70% formamide and 10 mM Tris-HCl, pH 7.5). The slides were then washed with PNA wash B for 5 min (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride (NaCl), and 0.8% Tween-20). Nuclear and chromosomal DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 0.5 µg/mL. The slides were rinsed twice with distilled water and air dried to remove excess water. Finally, the slides were mounted using ProLong Gold Antifade Mounting Medium (Invitrogen) and stored overnight before imaging. Images were acquired using a Zeiss LSM 800 confocal microscope with a 63X objective with airyscan module and 4% laser power with 1xbinning in super resolution mode. DAPI and Alexa Fluor 647 signals were captured using the appropriate excitation and emission settings. Metaphase spreads with clear chromosome separation were selected for telomere analysis²⁰¹.

4.22.2 Flow fluorescence *in situ* hybridisation (Flow-FISH)

Flow-FISH was performed to measure telomere associated fluorescence intensity in HeLa and MCF7 cells after treatment with TRFiN compounds. At the end of treatment, both floating and adherent cells were collected to avoid loss of detached cells. The cells were washed with PBS, centrifuged, and counted. Equal numbers of cells were used for each sample. For each treatment group, two parallel tubes were prepared, one with telomere PNA probe and one without probe for background fluorescence correction. The cells were fixed and permeabilised with cold 80% methanol (80 mL absolute methanol and 20 mL distilled water). The fixative was added dropwise to the cell pellet under gentle vortexing to reduce cell aggregation. The cells were incubated in fixative for 1 h at room temperature or overnight at 2-8 °C. After fixation, the cells were centrifuged and washed three times with PBS to remove residual methanol. The washed cell pellet was then resuspended in PNA hybridisation solution containing Alexa Fluor 647 labelled telomere PNA probe at a final concentration of 0.3 µg/mL (70% deionised formamide, 0.25% blocking reagent (Roche), and 10 mM Tris-HCl, pH 7.5). The probe free control was resuspended in the same hybridisation solution without PNA probe. The samples were incubated for 10 min at room temperature in the dark before denaturation. DNA denaturation was performed at 80 °C for 10 min. After denaturation, the samples were hybridised overnight at room temperature in the dark. After hybridisation, the cells were centrifuged and washed with PNA wash A for 10 min (70% formamide and 10 mM Tris-HCl, pH 7.5).

The wash with PNA wash A was repeated once to remove unbound probe. The cells were then washed with PNA wash B for 5-10 min (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride (NaCl), and 0.8% Tween-20). After the final wash, the cells were counterstained with DAPI at 0.5 µg/mL when DNA content based gating was required. The cells were resuspended in PBS and analysed using an Agilent NovoCyte flow cytometer. Alexa Fluor 647 telomere PNA fluorescence was detected using the appropriate far red fluorescence channel. Cell debris was excluded using forward scatter and side scatter gating. Cell aggregates and doublets were removed using pulse area and pulse width gating. Telomere fluorescence was measured in the single cell population. For each sample, the median fluorescence intensity of the probe free tube was subtracted from the corresponding PNA probe tube, and the corrected telomere fluorescence intensity was compared between control and TRFiN treated cells. At least 13,000 single cells were acquired for each sample²⁰².

4.23 Western blot analysis of protein expression

Western blot analysis was performed to examine the expression of selected proteins in HeLa and MCF7 cells after treatment with TRFiN compounds. Cells were seeded in complete culture medium in tissue culture plates at a density of 9.5×10^3 cells/cm². The cells were seeded in triplicate and allowed to attach before treatment. After attachment, cells were treated with TRFiN41, TRFiN49, TRFiN72, and the parent compound A822 at their respective IC₅₀ values for 168 h, as described previously. Cells treated with 1% DMSO were used as the vehicle control. After the required treatment period, cells were lysed directly in 1× Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol). The buffer was supplemented freshly with cOmplete Mini EDTA free Protease Inhibitor Cocktail and phosphatase inhibitor cocktail to preserve total and phosphorylated proteins. The cell lysates were collected and protein concentration was determined before gel loading. For each sample, 30 µg of total protein was separated using 4-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes (Bio-Rad). Polyvinylidene difluoride membranes were used when the same blot was required for stripping and reprobing. The membranes were blocked for 1 h at room temperature using 5% non-fat dry milk or 5% BSA in Tris buffered saline containing 0.1% Tween 20 (TBST). Tris buffered saline with Tween 20 contained 0.2 M Tris base, 0.137 M NaCl, and 0.1% Tween 20. For the detection of phosphorylated epitopes, membranes were blocked with BSA. For non-phosphorylated proteins, non-fat dry milk (Sigma-Aldrich) was used for blocking. After blocking, the membranes were incubated with the respective primary antibodies overnight at 4 °C. Details of all primary antibodies and their working dilutions are provided in the appendix. Following primary antibody incubation, the membranes were washed three times with TBST. The membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. After secondary antibody incubation, the membranes were washed again with TBST. Protein bands were detected using an enhanced chemiluminescence detection reagent kit (Thermo Fisher Scientific). Chemiluminescent signals were acquired using a Azure 300 Chemiluminescent Imaging System (Azure Biosystems). When detection of another protein was required on the same membrane, the blot was stripped using stripping buffer (Invitrogen) according to the manufacturer's instructions and reprobed with the required antibody. Band intensity was quantified using Image Lab 5.2 software (Bio-Rad). For each target protein, band intensity was first normalized to the corresponding beta-actin band from the same sample. The normalized values were then expressed as fold change relative to the corresponding untreated control, which was assigned a value of 1:

$$\text{Normalized target intensity} = \text{Target band intensity} / \text{beta-actin band intensity}; \text{Fold change} = \frac{\text{Normalized target intensity of treated sample}}{\text{Normalized target intensity of untreated control}}$$

Equal amounts of total protein (30 µg) were loaded in each lane. Beta-actin normalization was applied to each target band to account for residual loading and transfer variation. For phosphorylated checkpoint proteins, the phosphorylated to total protein ratios (pCHK1/CHK1, pCHK2/CHK2,

pATM/ATM, and pATR/ATR) were calculated and expressed relative to the untreated control. Lamin B1 was not used as a loading control because its treatment dependent change was itself considered a biologically relevant endpoint.

4.24 RNA sequencing library preparation and transcriptome data analysis

RNA sequencing was performed to examine the transcriptome level changes induced by TRFiN compounds in HeLa and MCF7 cells. The workflow included RNA isolation, quality assessment, library preparation, sequencing, read processing, differential gene expression analysis, functional enrichment, and data visualisation. The complete analysis was carried out separately for each cell line to compare the transcriptional response of each treatment group with the untreated control.

4.24.1 RNA isolation and quality assessment

Total RNA was isolated from HeLa and MCF7 cells maintained under five experimental conditions: untreated control, TRFiN41, TRFiN49, TRFiN72, and A822. Biological replicates were included for each condition. Total RNA was extracted from the cells using the Total RNA Zol Out™ Kit (A&A Biotechnology), following the manufacturer's protocol. An on column DNase treatment step was included during RNA isolation to reduce genomic DNA contamination. RNA quality and integrity were analysed using an Agilent 2100 Bioanalyzer and 2200 TapeStation (Agilent Technologies). RNA integrity number (RIN) values were recorded for all samples. Only high quality RNA samples with RIN values between 8 and 10 were used for library preparation.

4.24.2 Library preparation and sequencing

Library preparation was performed using the TruSeq Stranded mRNA kit (Illumina) according to the manufacturer's instructions. Library size distribution and fragment quality were checked using an Agilent 2100 Bioanalyzer with a DNA 1000 chip. The prepared libraries were quantified by quantitative polymerase chain reaction (qPCR) using the Illumina qPCR Quantification Protocol. The libraries were sequenced externally at Macrogen Europe, Amsterdam, The Netherlands, using an Illumina NextSeq platform. Sequencing was performed to generate 150 bp paired end reads for all samples.

4.24.3 RNA-seq read processing and alignment

Initial RNA sequencing data processing, including mapping and read count generation, was performed on the Tryton p supercomputer at Gdańsk University of Technology (CI TASK) using the nf-core RNA-seq workflow. Raw reads were first processed for adapter removal and quality trimming. Duplicate reads were filtered using fastp together with UMI-tools when applicable. Reads not originating from messenger RNA, including ribosomal RNA and other contaminant sequences, were removed using BBSplit and SortMeRNA. The cleaned reads were aligned to the human reference genome GRCh38 using STAR. Gene level and transcript level quantification was performed using RNA Seq by Expectation Maximization (RSEM). Mapping statistics and duplication related metrics were checked using SAMtools and dupRadar.

4.24.4 Differential gene expression analysis

Downstream analysis was performed in R version 4.5.2 using RStudio. RSEM expected counts were combined into gene level count matrices separately for HeLa and MCF7 cells. A sample annotation file was prepared to assign each sample to its respective cell line and treatment group. The treatment groups included untreated control, TRFiN41, TRFiN49, TRFiN72, and A822, with untreated control used as the reference group. Differential gene expression analysis was performed separately for each cell line using DESeq2. The design formula modelled gene expression as a function of treatment. Genes with very low abundance were removed before differential expression analysis. Genes with a total count below 10 across all samples were excluded. Counts per million (CPM) values were calculated using edgeR, and genes were considered expressed when CPM was ≥ 1 in at least two

samples. For each treatment versus control comparison, DESeq2 was used to estimate log₂ fold change values and Wald test P values. The P values were adjusted using the Benjamini-Hochberg method. Differentially expressed genes were defined as genes with an adjusted P value ≤0.05 and an absolute log₂ fold change ≥1.

4.24.5 Functional enrichment analysis

Functional analysis was performed to identify biological processes and pathways associated with the differentially expressed genes. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were carried out using clusterProfiler based tools. Human gene annotations were used, and Entrez Gene identifiers were assigned during the annotation step. Gene set enrichment analysis was also performed using pre-ranked gene lists prepared for each comparison. The genes were ranked according to log₂ fold change values and analysed using curated Gene Ontology and Kyoto Encyclopedia of Genes and Genomes gene sets.

4.24.6 Data visualisation

Global expression patterns and sample clustering were examined after variance stabilising or regularised log transformation of normalised counts. Principal component analysis was performed using the transformed count data. Boxplots and density plots were used to examine the distribution of normalised expression values across samples. Volcano plots were generated for each treatment versus control comparison to visualise log₂ fold change against $-\log_{10}$ P values. The most strongly regulated genes, including approximately 10 up regulated and 10 down regulated genes, were labelled using Human Genome Organisation gene symbols. All shell scripts and R scripts used in the analysis workflow, including scripts for differential expression analysis, enrichment analysis, gene set enrichment analysis, and plot generation, are provided in the Appendix. The complete workflow, supporting diagrams, and data files are provided in the supplementary drive submitted with the thesis.

4.25 Software, computational tools and statistical analysis

Statistical analysis was performed using GraphPad Prism 10¹⁸⁴ unless stated otherwise. Data obtained from independent experiments were entered into GraphPad Prism 10 for calculation of mean values, standard deviation or standard error of the mean, and statistical comparison between experimental groups. Depending on the experimental design, statistical significance was analysed using Student's t test or analysis of variance (ANOVA). Some statistical calculations and graphical outputs were also generated in R version 4.5.2²⁰³ using RStudio²⁰⁴. Flow cytometry data acquired using the Agilent NovoCyte flow cytometer were analysed using FlowJo v10²⁰⁵ and Novo Express software²⁰⁶. The quantified flow cytometry values were exported to GraphPad Prism 10 for statistical analysis and graph preparation. Western blot band intensity was quantified using Image Lab 5.2 software (Bio-Rad)²⁰⁷. Microscopy images and DNA fibre lengths, colocalisation were analysed using ImageJ software²⁰⁸. Spheroid growth and live cell imaging measurements were analysed using Olympus CellSens software²⁰⁹. Time lapse wound healing images were assembled using CellSens software²⁰⁹, and wound gap area, wound width, and migration velocity were calculated using ImageJ²⁰⁸. Surface plasmon resonance data obtained from Biacore T200 were analysed using Biacore T200 Evaluation Software (Cytiva)²¹⁰. RNA sequencing data processing was performed on the Tryton p supercomputer at Gdańsk University of Technology (CI TASK) using the nf-core RNA-seq workflow²¹¹. The RNA sequencing workflow included fastp, UMI-tools, BBSplit, SortMeRNA, STAR, RSEM, SAMtools, and dupRadar for read processing, alignment, quantification, and quality assessment. Differential gene expression and downstream analysis were performed in R using DESeq2, edgeR, clusterProfiler based tools, and gene set enrichment analysis. CAM image analysis was performed using an in house Python script developed for quantification of vascular growth from

chorioallantoic membrane images. Molecular docking studies were performed using Schrödinger Maestro modules^{212,213}. Molecular dynamics simulations were performed using Desmond²¹⁴. All shell scripts, R scripts, Python scripts, computational workflows, supporting diagrams, and related data files are provided in the supplementary drive attached with the thesis.

Chapter 5

Results

5.1 Selection of lead compounds for biological evaluation

The compounds used in the present study were selected through a virtual screening workflow designed to identify promising small molecules capable of targeting the TRF1-TIN2 interaction interface. The chemical structures of all compounds are provided in the Appendix. For this purpose, structural variations were generated by considering different substituent groups at four positions of the parent scaffold, compound A822. The selection of these groups was based on chemical availability with respect to synthesis, and also on the local environment of the original TIN2 position, in order to explore potential interactions with the surrounding amino acid residues. In the case of R1 and R2, 39 different R groups were considered, while for R3, 29 synthetically possible groups were selected. In addition, 16 different structures were considered for R4. These analogues were generated using the R group enumeration wizard available in Schrödinger Maestro (Maestro v12.8). By this approach, a total of 18,096 structures were enumerated and subjected to docking analysis. All enumerated compounds were docked against the crystal structure of the TRF1-TRFH domain in complex with the TIN2 peptide (PDB ID: 3BQO), which was used as the target structure for the virtual screening study. The aim of this workflow was to identify compounds with favourable binding at the TIN2 binding site of TRF1. From the total number of screened structures, a smaller group of compounds showed proper binding within the target pocket at TRF1 binding TIN2 interface. Among these, TRFiN33, TRFiN38, TRFiN41, TRFiN42, TRFiN43, TRFiN44, TRFiN45, TRFiN47, TRFiN49, TRFiN72, and TRFiN67b compounds were found to bind well at the TIN2 binding site of the protein and were therefore selected for further consideration (Figure 5.1B-D). Within this compound set, A822 was treated as the main lead compound and served as the parent scaffold for comparison throughout the study. Along with A822, its derivatives, namely TRFiN33, TRFiN38, TRFiN41, TRFiN42, TRFiN43, TRFiN44, TRFiN45, TRFiN47, TRFiN49, TRFiN72, and TRFiN67b, were included for biological evaluation. All these derivatives were synthesised in a separate study by other members of the project. The structures of A822 and its selected derivatives are presented in Figure 5.1A. The inclusion of both the parent compound and its derivatives made it possible to compare the biological behaviour of a focused series of structurally related molecules rather than assessing only a single lead compound. This was important for the present study because it provided an initial basis for understanding how small structural changes influenced biological response. Since A822 was considered the main reference compound, the activity of the derivative series could be compared directly with it. Such an approach was useful in the early stage of lead evaluation, as it allowed the identification of derivatives showing either comparable or improved effects in the tested systems. It also helped to narrow the series to the most relevant molecules for subsequent experimental validation. As the chemical synthesis of these compounds was carried out as part of a separate research project, the present thesis does not describe the synthetic route in detail. Instead, the emphasis here is on the biological evaluation of the selected compounds and their relevance to the proposed mechanism of action. Thus, the virtual screening workflow served as the starting point for selecting the most promising molecules, while the experimental work described in the following sections was aimed at examining their biological effects in suitable cancer models. It is worth noting that due to the limited size of A822 and its derivatives

only the part of the TIN2 binding cleft could be explored (left binding pocket visible on Figure 5.1C). Anyway both the hit compound A822 and its derivatives occupy this part (Figure 5.1D) quite well.

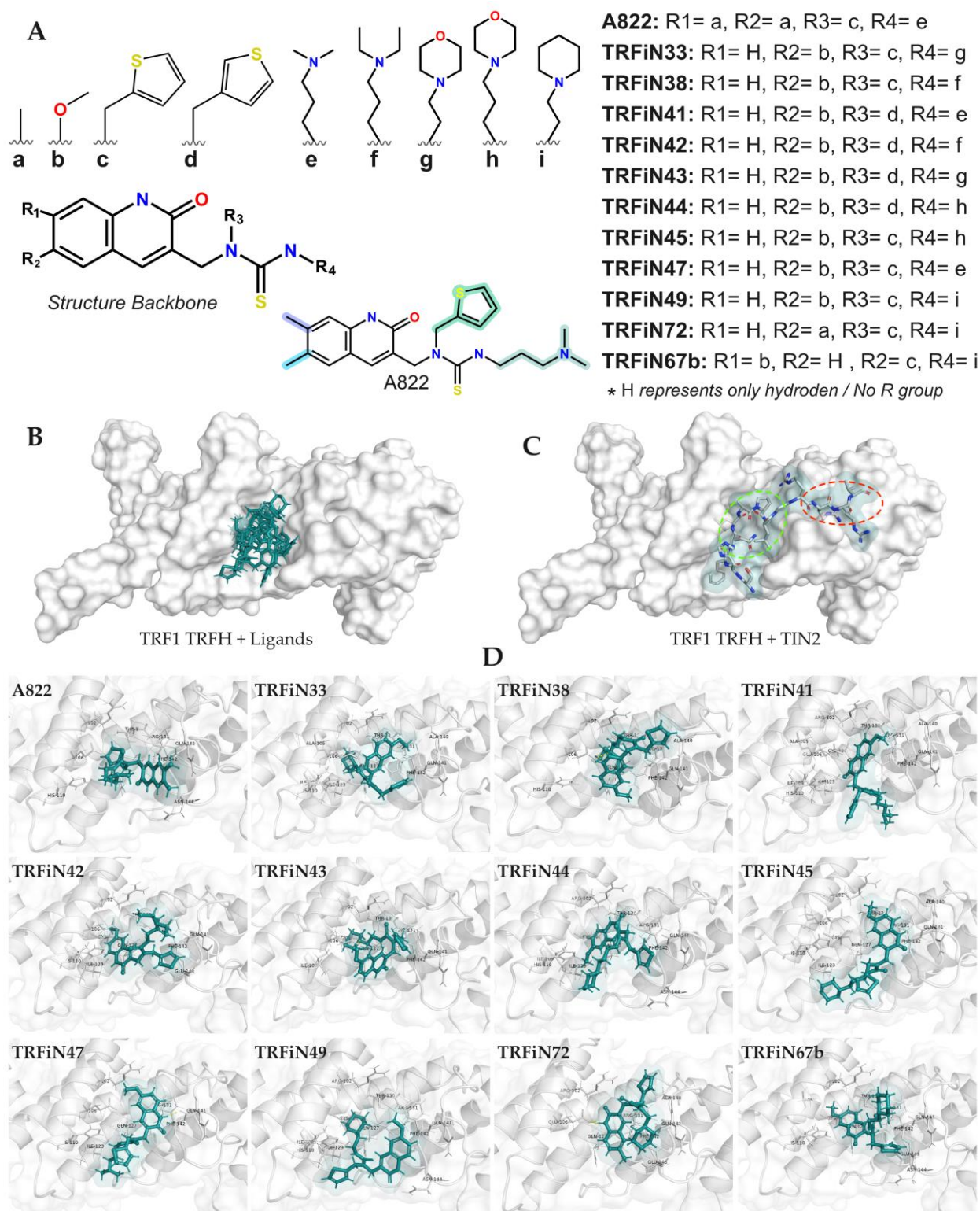


Figure 5.1. Virtual screening based selection of A822 and its derivatives targeting the TRF1-TIN2 interaction site. (A) Structures of the parent compound A822 and selected derivatives generated by R group enumeration and chosen for biological evaluation after virtual screening against the TRF1-TIN2 interface. (B) Overlay of the docked poses of the selected ligands in the TRF1 TRFH binding pocket. (C) Crystal structure of the TRF1 TRFH domain in complex with the TIN2 peptide (PDB: 3BQO) used for docking. The native position of the TIN2 binding site is marked in colour. (D) Individual binding poses of A822 and its derivatives at the TIN2 binding site of TRF1.

5.2 Cytotoxicity and selectivity profile of A822 and its derivatives

The cytotoxicity screening showed considerable differences in the activity of A822 and its derivatives across the cancer cell line panel. This variation was evident in the IC₅₀ and SI heat maps presented in Figure 5.2A,B. When the median IC₅₀ was calculated across the 19 cancer cell lines, 4OHT was the most potent reference compound, with a median value of 2.64 μ M. Among the synthesised compounds, TRFiN72 showed the highest activity, with a median IC₅₀ of 6.12 μ M, which was close to the value of 6.35 μ M obtained for the parent compound A822. TRFiN67b and TRFiN49 also showed favourable activity, with median values below 9 μ M. In contrast, TRFiN33, TRFiN45, TRFiN44, and TRFiN43 showed median IC₅₀ values between 30.46 and 37.03 μ M. These values were approximately five- to six fold higher than that of TRFiN72, indicating markedly weaker cytotoxic activity. TRFiN41 showed an intermediate profile, with a median IC₅₀ of 16.08 μ M. The screening therefore separated the derivatives into a low micromolar active group, an intermediate group, and a group with considerably weaker activity.

The selectivity of the compounds was evaluated using normal cells corresponding to the tissue origin of each cancer group. Human mammary epithelial cells (HMEC) were used for the breast cancer panel, CCD 841 CoN cells for the colon cancer panel, and MRC-5 fibroblasts for the remaining cancer cell lines. The SI was calculated by dividing the IC₅₀ in the corresponding normal cells by the IC₅₀ in each cancer cell line. An SI above 1 indicated that the cancer cells were more sensitive than the normal cells, whereas an SI below 1 indicated similar or greater sensitivity of the normal cells. This tissue matched comparison showed that high cytotoxic activity did not always correspond to favourable selectivity. Within the breast cancer panel, TRFiN72, TRFiN67b, and TRFiN49 produced a closely related low micromolar response across hormone responsive, hormone independent, and tamoxifen resistant cell lines. Their combined IC₅₀ values were mainly between approximately 4 and 12 μ M, although TRFiN49 was slightly less active in BT-474 and MDA-MB-231 cells. A822 was also active in the breast cancer panel, but its response was less uniform. It showed stronger activity in MCF7 and T-47D cells and weaker activity in BT-20 and tamoxifen resistant MCF7 Tam1 cells, resulting in an IC₅₀ range of 4.80-14.69 μ M. The difference between these compounds became clearer when their effects on HMEC cells were considered. TRFiN72 and TRFiN49 were substantially less toxic to HMEC cells than to most of the breast cancer cells, whereas HMEC cells remained highly sensitive to A822.

This difference was reflected in the breast cancer SI values. TRFiN72 showed a median SI of 5.18, indicating an approximately five fold separation between its activity in breast cancer cells and HMEC cells. TRFiN49 showed the next most favourable value at 3.07, while the median SI of TRFiN67b was 1.76. In contrast, A822 had a median SI of only 0.52 because its IC₅₀ in HMEC cells was lower than the values obtained in several breast cancer cell lines. TRFiN72 therefore showed the best balance between cytotoxic activity and breast cell selectivity, followed by TRFiN49. Although TRFiN67b remained active, its separation between cancer and normal breast cells was more limited. The similar activity ranges of these three derivatives may reflect related cellular responses, but cytotoxicity patterns alone cannot confirm that they act through the same molecular mechanism. A similar activity pattern was observed in the colon cancer panel. A822 was highly active against HCT116, HT29, HT29-MTX, and LoVo cells, with IC₅₀ values between 4.63 and 9.41 μ M. TRFiN72 remained within a comparable range, while TRFiN67b and TRFiN49 showed slightly weaker activity in some colon cancer cell lines. However, the high potency of A822 was not accompanied by colon cell selectivity. The IC₅₀ of A822 in normal CCD 841 CoN cells was only 0.61 μ M, showing that these cells were more sensitive to the parent compound than any of the tested colon cancer cells. As a result, A822 produced a median colon SI of only 0.10. TRFiN72 showed a median SI of 2.00, whereas TRFiN67b and TRFiN49 showed values of 1.43 and 1.40, respectively. The colon cancer results therefore followed the pattern observed in the breast panel, with TRFiN72 showing the most favourable balance between activity in cancer cells and reduced effects on the tissue matched normal cells.

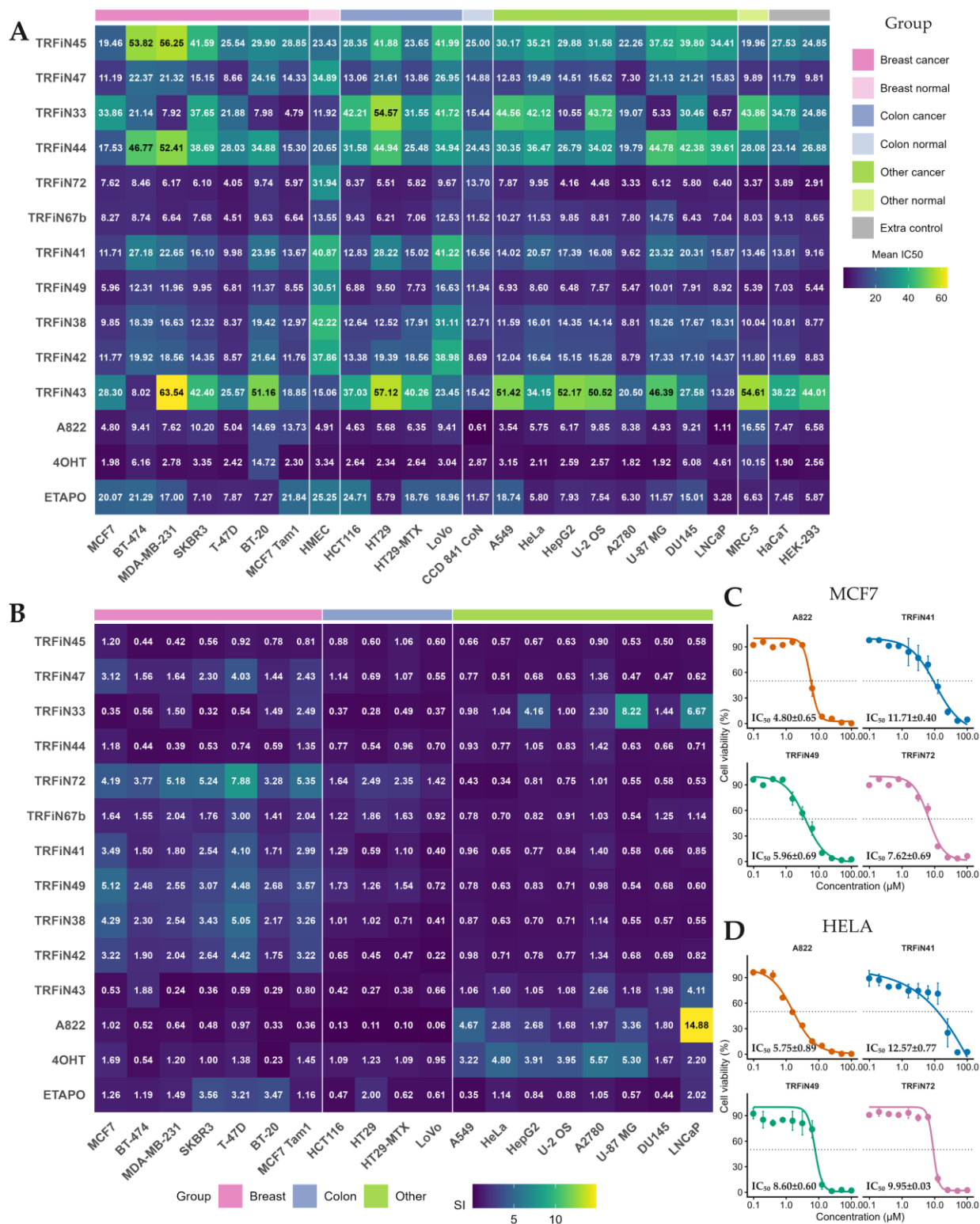


Figure 5.2. Heat map analysis of IC_{50} values and selectivity index together with representative dose response curves for A822 and selected TRFiN derivatives. (A) Heat map showing IC_{50} values (μ M) of the tested compounds in breast cancer, colon cancer, other cancer, normal, and extra control cell lines. (B) Heat map showing selectivity index (SI) values calculated relative to the corresponding normal cell lines. (C) Dose dependent inhibition of A822, TRFiN41, TRFiN49, and TRFiN72 in MCF7 cells. (D) Dose dependent inhibition of A822, TRFiN41, TRFiN49, and TRFiN72 in HeLa cells. All IC_{50} values are shown as mean $\pm$ SD from three independent experiments. The IC_{50} values together with the corresponding SD values are provided in Appendix.

Across the eight remaining cancer cell lines, A822 and the three active derivatives generally maintained low micromolar activity, although the response varied according to cell type. A822 was particularly active in LNCaP prostate cancer cells, where its IC_{50} was 1.11 μ M. TRFiN72 showed strong

activity in A2780, HepG2, and U-2 OS cells, while TRFiN49 showed a narrower activity range across this mixed cancer group. The comparison with MRC-5 fibroblasts produced a different selectivity pattern. MRC-5 cells were highly sensitive to TRFiN72 and TRFiN49, resulting in median SI values below 1 for TRFiN72, TRFiN67b, and TRFiN49 across the eight cancer cell lines. A822 was less toxic to MRC-5 cells and produced a median SI of 2.78 in this group. Its strongest separation was observed in LNCaP cells, where the SI reached 14.88. The favourable selectivity of TRFiN72 in the breast and colon panels therefore did not extend to the MRC-5 comparison. The additional HaCaT and HEK-293 control cell lines were also sensitive to several derivatives, particularly TRFiN72, further showing that selectivity depended on the normal cell model used for comparison.

The reference drugs provided a comparative range for interpreting the compound series. In most cancer cell lines, 4OHT showed IC₅₀ values between 1.82 and 6.16 μM . BT-20 cells were a clear exception, with a considerably higher IC₅₀ of 14.72 μM . Etoposide showed a broader activity range of 3.28–24.71 μM and was generally less active than 4OHT and the strongest TRFiN derivatives. Within the synthesised series, TRFiN72 had the strongest initial screening profile because it combined low micromolar activity with the highest median SI in the breast and colon panels. TRFiN49 and TRFiN67b formed the remaining part of the active subgroup, although their selectivity was lower. A822 remained an important parent compound because of its strong cytotoxicity in several cancer cell lines, particularly LNCaP, but its high activity against HMEC and CCD 841 CoN cells reduced its advantage in the breast and colon comparisons. The complete IC₅₀ values with their standard deviations are provided in Appendix.

5.3 SPR analysis of TRF1 binding and selection of lead compounds for further biological evaluation

The direct interactions of A822 and its derivatives with TRF1 were examined by SPR. The MTT screening identified TRFiN41, TRFiN42, TRFiN49, TRFiN38, TRFiN67b and TRFiN72 as the most active derivatives in this series, as these compounds showed a similar antiproliferative pattern across multiple cancer cell lines (Figure 5.2A). This repeated trend suggested that these derivatives may act through a common biological mechanism. Since the compounds were designed to interfere with the TRF1-TIN2 interaction in the shelterin complex, the similar response seen in different cancer cell lines is in agreement with the proposed mode of action. For the next stages of the study, MCF7 and HeLa cells were selected for further experiments. These cell lines were chosen based on previous standardised studies in telomere and shelterin research. They are also easy to culture, show good proliferation under routine conditions, and are less expensive to maintain for long experiments. For these reasons, MCF7 and HeLa were used as suitable models for further biological evaluation.

The direct binding of the parent compound and its derivatives to TRF1 was then examined by surface plasmon resonance. The SPR sensorgrams are shown in Figure 5.3, and the calculated kinetic parameters are listed in Table 5.1. The results showed that the parent compound A822 and several of its derivatives were able to bind directly to TRF1. The previously in house synthesised TIN2 peptide⁵⁷ was included as a reference ligand because it is the native binding partner of TRF1 in this system. Since the small molecules were tested in the μM range and TIN2 was tested in the nM range, the concentrations used in the assay cannot be compared directly. A more useful comparison is given by the calculated binding constants.

A822 and most of the TRFiN derivatives produced concentration dependent SPR responses, although the maximum observed RU differed considerably among the compounds. TRFiN49 produced the highest response among the small molecules at approximately 202 RU, while A822 and TRFiN72 produced maximum responses of approximately 24 and 72 RU, respectively. TRFiN38, TRFiN41, and TRFiN42 also produced relatively high responses despite having weaker kinetic affinities. The variation between response magnitude and the equilibrium dissociation constant (KD) showed that surface accumulation and binding affinity did not follow the same order across the compound series. A822 produced the lowest fitted kinetic KD among the tested small molecules. Its association rate constant (k_a) was $1.06 \times 10^{-5} \text{ nM}^{-1} \text{ s}^{-1}$, and its dissociation rate constant (k_d) was

$6.16 \times 10^{-3} \text{ s}^{-1}$, giving a kinetic K_D of 580.35 nM or 0.58 μM . This kinetic profile was observed despite its relatively low maximum response of 24.49 RU. The steady state K_D of A822 was 21.6 μM , which was higher than its kinetic value. Thus, A822 showed the most favourable kinetic K_D , while its steady state analysis placed the interaction within the micromolar range.

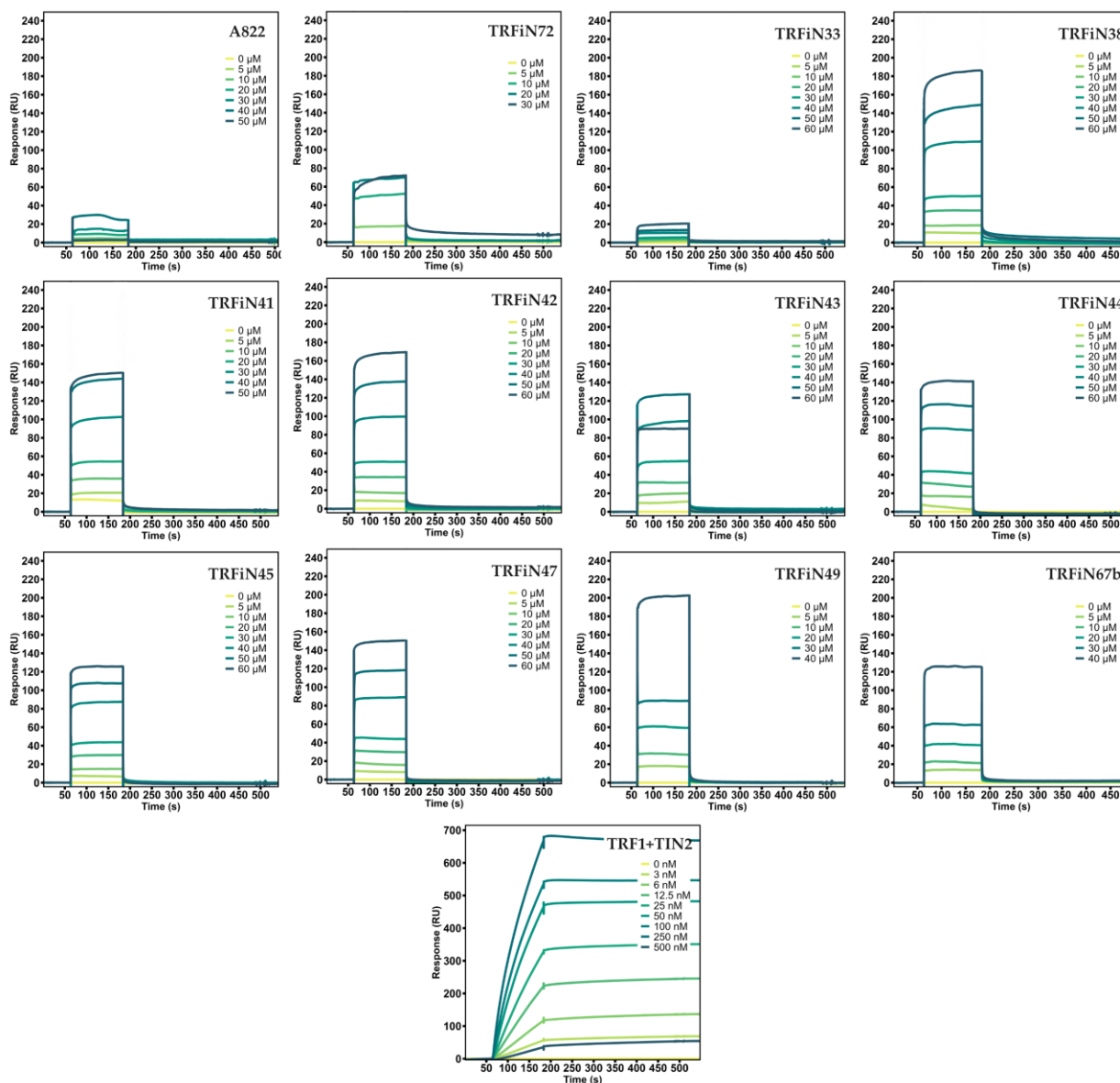


Figure 5.3. SPR sensorgrams of A822, TRFiN derivatives, and the TIN2 peptide binding to TRF1. The sensorgrams show the direct interaction of the parent compound A822, its derivatives, and the reference TIN2 peptide with TRF1 under the tested conditions. The binding curves were used to calculate the association rate constant (k_a), dissociation rate constant (k_d), kinetic dissociation constant (K_d), and steady state K_D , as summarised in Table 5.1.

Among the derivatives, TRFiN49 produced the lowest kinetic K_D at 6.83 μM and was the strongest derivative according to the kinetic analysis. It also generated the highest maximum response among the small molecules. TRFiN67b and TRFiN38 followed with kinetic K_D values of 14.48 and 19.89 μM , respectively. TRFiN72 and TRFiN45 showed comparable kinetic K_D values of 24.58 and 25.75 μM . The steady state K_D of TRFiN72 was 15.4 μM and remained within the same micromolar range as its kinetic value. TRFiN72 therefore showed the closest agreement between the two affinity measurements. TRFiN33 and TRFiN42 were weaker, with kinetic K_D values of approximately 50 μM . TRFiN41 produced a relatively high response of 150.31 RU but showed the weakest valid kinetic affinity, with a K_d of 77.89 μM . This result further showed that the magnitude of the SPR response was not directly related to the calculated binding affinity.

TRFiN43, TRFiN44, and TRFiN47 produced measurable SPR responses, but physically meaningful association rate constants could not be obtained. Their kinetic KD values were therefore reported as not available. The steady state KD values marked with double asterisks were mathematically obtained from curve fitting because a clear binding plateau was not reached and were excluded from direct affinity ranking. Based on the valid kinetic values, the derivative affinity order was TRFiN49, TRFiN67b, TRFiN38, TRFiN72, TRFiN45, TRFiN33, TRFiN42, and TRFiN41. The TIN2 peptide produced a substantially higher maximum response of approximately 630 RU and showed a steady state KD of 9.85 nM. This value indicated stronger binding than that observed for the small molecules and was consistent with the native interaction between TIN2 and TRF1. The kinetic KD of the peptide was 6.44 μ M, which differed considerably from the steady state result. The large difference between the kinetic and steady state values suggests that the peptide interaction was not described well by the simple kinetic model used for the compounds. For this reason, the steady state KD is the more meaningful value for TIN2 in the present dataset. Based on this value, TIN2 bound TRF1 much more strongly than any of the tested small molecules. This may be due to the smaller surface occupied at the TRF1 interface by the small molecules studied compared with the native TIN2 peptide.

Table 5.1. SPR binding parameters of A822, TRFiN derivatives, and TIN2 peptide against TRF1

Compound	k_a ($\text{nM}^{-1} \text{s}^{-1}$)	k_d (s^{-1})	KD, kinetic (nM)	KD, steady state (nM)	RU (maximum response)
TRFiN67b	4.39×10^{-6}	6.358×10^{-2}	1.45×10^4	$2.39 \times 10^{11**}$	125.485
TRFiN38	7×10^{-7}	1.393×10^{-2}	1.99×10^4	$2.05 \times 10^{12**}$	186.124
TRFiN41	2.56×10^{-7}	1.991×10^{-2}	7.79×10^4	$3.54 \times 10^{15**}$	150.311
TRFiN42	7.19×10^{-7}	3.651×10^{-2}	5.08×10^4	$7.32 \times 10^{11**}$	169.233
TRFiN43	NA	3.393×10^{-2}	NA	$2.44 \times 10^{5**}$	127.063
TRFiN44	NA	NA	NA	$3.1 \times 10^{12**}$	141.104
TRFiN45	1.31×10^{-6}	3.366×10^{-2}	2.58×10^4	$5.03 \times 10^{15**}$	125.679
TRFiN47	NA	8.099×10^{-2}	NA	$1.25 \times 10^{12**}$	150.4
TRFiN49	2.39×10^{-6}	1.635×10^{-2}	6.83×10^3	$5.75 \times 10^{12**}$	202.341
TRFiN72	1.03×10^{-6}	2.529×10^{-2}	2.46×10^4	1.54×10^4	71.8301
TRFiN33	4.26×10^{-7}	2.115×10^{-2}	4.96×10^4	$4.85 \times 10^{11**}$	20.584
A822	1.06×10^{-5}	6.16×10^{-3}	5.803452×10^2	2.16×10^4	24.4922
TIN2	1.12×10^{-6}	7.23×10^{-3}	6.44×10^3	9.84658	630.199

Note: NA, not obtained because the fitted k_a value was negative or non-physical. **, mathematically derived KD, since a clear binding plateau was not reached within the tested concentration range; (k_a) association rate constant, (k_d) dissociation rate constant, (K_d) kinetic dissociation constant, (KD) steady state, and (RU) maximum response unit.

5.4 γ H2AX induction and TIN2- γ H2AX colocalisation after treatment with A822 and TRFiN derivatives

The flow cytometric histograms in Figure 5.4A showed clear shifts in γ H2AX fluorescence after 168 h of treatment with A822, TRFiN41, TRFiN49, and TRFiN72. In contrast, the distributions of the weaker derivatives remained closer to the untreated control. Quantification of the γ H2AX positive populations in Figure 5.4B showed that MCF7 control cells contained only $2.59 \pm 0.57\%$ positive cells. TRFiN41 and TRFiN72 produced the strongest responses, increasing the γ H2AX positive population to approximately 45-47%. These values represented about 18-fold increases over the control. A822 and TRFiN49 produced values of approximately 23%, corresponding to increases of about nine fold, while TRFiN67b produced an intermediate response of $13.92 \pm 2.95\%$. The remaining derivatives produced only 3.00-4.29% γ H2AX positive cells and remained close to the control population. A stronger response was detected in HeLa cells, where the control contained $3.65 \pm 0.77\%$ positive cells. A822 and TRFiN72 increased the γ H2AX positive population to approximately 73-74%, while TRFiN41 and TRFiN49 produced values of approximately 63%. These responses represented increases of approximately 17- to 20-fold over the control. TRFiN67b increased the positive population to $25.62 \pm 3.40\%$, whereas the remaining derivatives produced values between 3.39 and 7.24%. The percentage of γ H2AX positive cells produced by each of the five responsive compounds was higher in HeLa than

in MCF7 cells, with the largest differences observed for A822 and TRFiN49. Nevertheless, the same compound groups were separated in both cell lines, with A822, TRFiN41, TRFiN49, and TRFiN72 producing the main response, TRFiN67b producing an intermediate response, and the remaining derivatives producing only small changes.

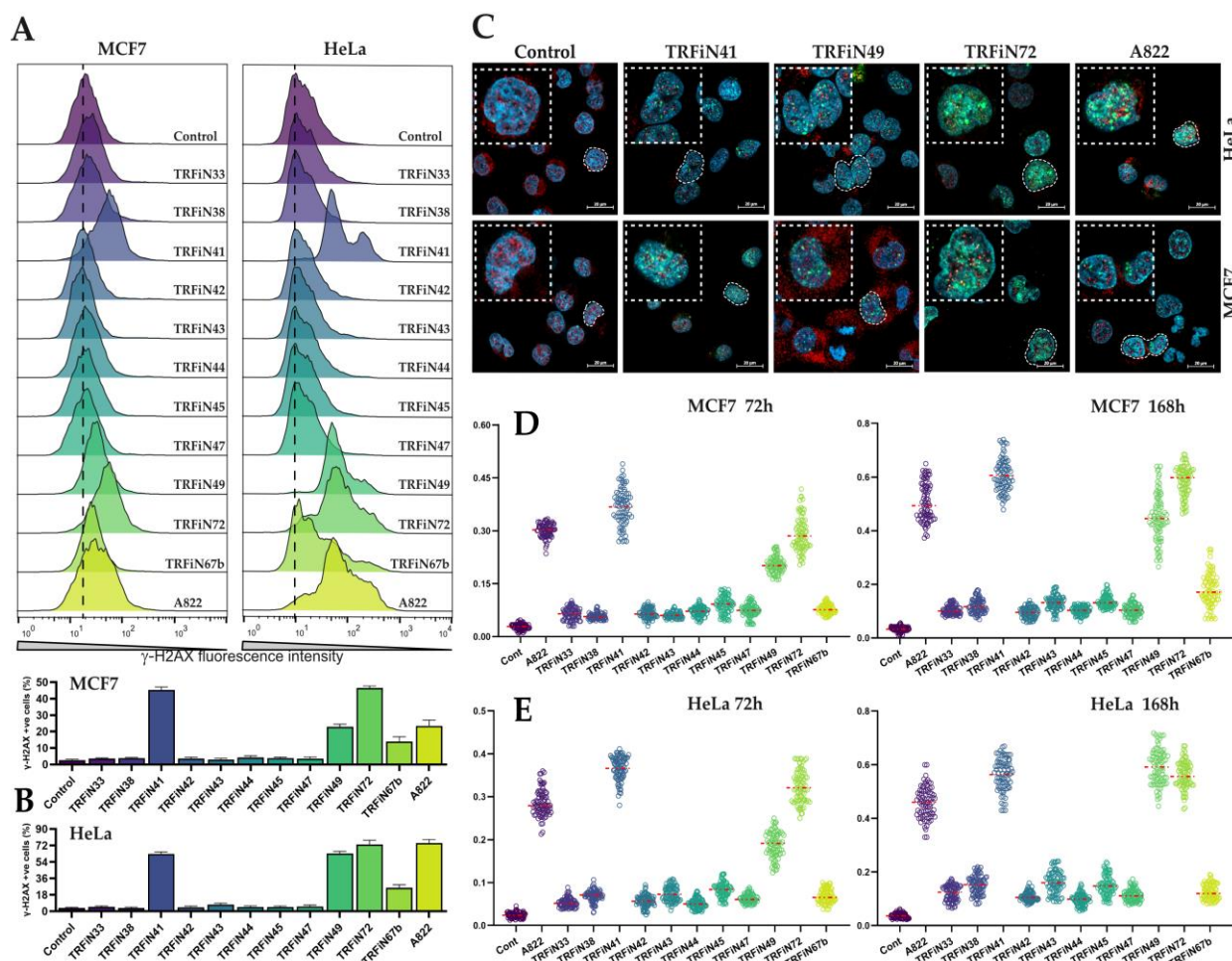


Figure 5.4. γ H2AX induction and TIN2- γ H2AX colocalisation after treatment with A822 and TRFiN derivatives. (A) Representative flow cytometric histograms of MCF7 and HeLa cells after 168 h treatment, and (B) percentage of γ H2AX positive cells at the same time point. (C) Representative colocalisation images of TIN2 and γ H2AX in untreated control, A822, TRFiN41, TRFiN49, and TRFiN72 treated MCF7 and HeLa interphase nuclei, while (D) and (E) show the Pearson correlation coefficient (PCC) values for MCF7 and HeLa after 72 h and 168 h treatment, respectively. All values are presented as mean $\pm$ SD, $n=4$.

The representative merged images in Figure 5.4C showed increased spatial overlap between TIN2 and γ H2AX signals following treatment with A822, TRFiN41, TRFiN49, and TRFiN72. Control nuclei showed limited overlap between the two signals, while treated nuclei contained a greater number of regions in which the TIN2 and γ H2AX fluorescence signals were present at the same location. This overlap was quantified using the Pearson correlation coefficient (PCC), with the MCF7 and HeLa measurements presented in Figure 5.4D and E, respectively. After 72 h, the control PCC values remained low at 0.029 ± 0.008 in MCF7 and 0.025 ± 0.007 in HeLa cells. Treatment with the four active compounds increased the PCC to 0.204-0.368 in MCF7 and 0.188-0.366 in HeLa cells, corresponding to approximately 7.0- to 14.6-fold increases over the respective controls. TRFiN41 produced the highest PCC in both cell lines at this time point. A822 and TRFiN72 also produced clear increases, with their relative positions differing only slightly between the two cell lines, while TRFiN49 showed the lowest PCC within the active group. TRFiN67b produced lower PCC values of 0.078 ± 0.014 in MCF7 and 0.068 ± 0.016 in HeLa cells. The remaining derivatives also stayed within a low range, reaching only 0.058-0.091 in MCF7 and 0.051-0.084 in HeLa cells. The difference between the active and weaker compound groups was therefore already visible after 72 h, even though the overall

γ H2AX positive population was quantified at the later 168 h time point. The PCC distributions at 168 h in Figure 5.4D and E showed a further increase in TIN2- γ H2AX colocalisation following treatment with the active compounds. In MCF7 cells, the control PCC remained low at 0.033 ± 0.009 , whereas A822, TRFiN41, TRFiN49, and TRFiN72 produced values between 0.450 and 0.607. TRFiN41 produced the highest PCC at 0.607 ± 0.061 , followed by TRFiN72 at 0.585 ± 0.056 , A822 at 0.506 ± 0.072 , and TRFiN49 at 0.450 ± 0.089 . In HeLa cells, the control PCC was 0.037 ± 0.009 , while the active compounds produced values between 0.459 and 0.592. TRFiN49 showed the highest PCC at 0.592 ± 0.065 , followed by TRFiN41 and TRFiN72, which produced closely related values of approximately 0.56. A822 produced a lower but still clearly increased PCC of 0.459 ± 0.057 . Compared with the 72 h measurements, TRFiN49 increased from 0.204 to 0.450 in MCF7 cells and from 0.188 to 0.592 in HeLa cells, while TRFiN72 increased from 0.292 to 0.585 in MCF7 cells and from 0.325 to 0.561 in HeLa cells. Across the four active treatments, the 168 h PCC values were approximately 1.5- to 3.2-fold higher than the corresponding 72 h values. TRFiN67b also increased with treatment time but remained below the active group, reaching 0.180 ± 0.062 in MCF7 and 0.126 ± 0.028 in HeLa cells. The remaining derivatives retained comparatively low PCC values at 168 h, ranging from 0.096 to 0.137 in MCF7 and from 0.098 to 0.162 in HeLa cells.

5.5 Molecular dynamics analysis of the TRF1-TRFH domain in apo and ligand bound states

To examine the effect of ligand binding on both the protein and the bound scaffold, molecular dynamics simulations were performed for the TRF1-TRFH domain from the crystal structure 3BQO in complex with A822, TRFiN41, TRFiN49 and TRFiN72, together with the unbound protein for principal component and free energy landscape analysis. Each simulation was run for 1000 ns under periodic boundary conditions in explicit solvent. A822 was used as the parent scaffold, while TRFiN41, TRFiN49 and TRFiN72 were selected derivatives. Structural stability was first assessed by RMSD, followed by protein ligand contact analysis and MM/GBSA binding free energy estimation. To further examine protein motion, principal component analysis (PCA) and free energy landscape (FEL) analysis were carried out using PC1 and PC2 as reaction coordinates. The RMSD profiles showed that the protein backbone remained stable in all ligand bound systems during the 1000 ns simulation period (Figure 5.5A). The mean C α RMSD values were 2.83 Å for the A822 complex, 3.13 Å for the TRFiN41 complex, 3.40 Å for the TRFiN49 complex and 2.80 Å for the TRFiN72 complex. The corresponding maximum values were 4.31, 4.73, 5.07 and 4.61 Å, respectively. These values show that the TRF1-TRFH domain retained its folded structure in all four complexes and did not undergo major structural drift during the simulation. The differences between the systems were small at the protein level, which means that the main variation between complexes was not caused by large scale unfolding or collapse of the protein domain. A stronger difference was observed when ligand RMSD was examined (Figure 5.5A). The parent scaffold A822 showed the highest ligand movement, with a mean ligand RMSD of 12.21 Å and a maximum value of 17.05 Å. This behaviour suggests marked repositioning of the ligand within the binding region during the simulation. In contrast, TRFiN41 showed the lowest ligand RMSD, with a mean value of 3.62 Å and a maximum of 6.72 Å, indicating that this derivative retained a much more stable binding pose through the simulation period. TRFiN49 and TRFiN72 showed intermediate behaviour, with mean ligand RMSD values of 6.35 and 5.72 Å, respectively. The difference between A822 and the derivatives is notable because the protein RMSD values remained in a narrow range, while the ligand RMSD values changed strongly across the series. This means that modification of the parent scaffold mainly affected how the ligand was retained in the binding site rather than how the protein domain itself behaved.

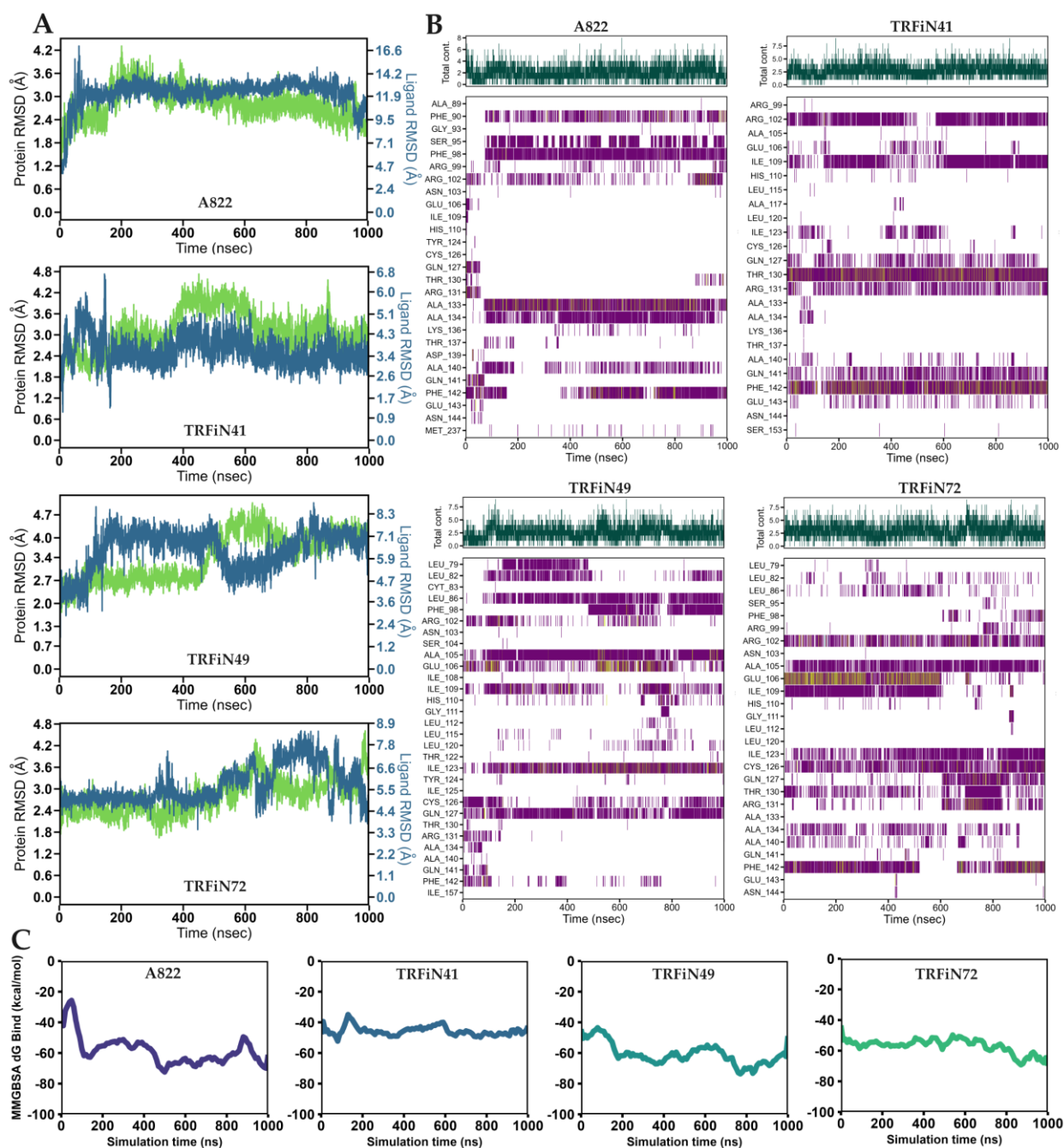


Figure 5.5 Molecular dynamics analysis of the TRF1-TRFH domain (3BQO) in complex with A822, TRFiN41, TRFiN49 and TRFiN72 over 1000 ns. (A) RMSD profiles of the protein backbone and bound ligand for each complex during the simulation. (B) Protein ligand contact maps showing the total number of contacts formed during the trajectory and the residue wise interaction pattern across simulation frames, including hydrogen bonds, hydrophobic contacts, ionic interactions, water bridges and halogen bonds. (C) MM/GBSA binding free energy profiles calculated along the simulation trajectory.

The high ligand RMSD of A822 suggests that the parent scaffold was able to remain associated with the site but did not maintain one fixed orientation over 1000 ns. Such movement may reflect partial reorientation within the pocket, repeated adjustment of substituent groups, or a less optimal fit against the local residue environment. This may also reflect the torsion profile of the ligands. By contrast, the much lower ligand RMSD of TRFiN41 points to a more persistent binding mode. TRFiN49 and TRFiN72 remained between these two extremes, which suggests that both derivatives improved ligand retention relative to A822 but still allowed some repositioning during the simulation. This pattern fits well with the idea that changes introduced on the A822 scaffold altered the way the compounds engaged the TRF1-TRFH surface. The protein ligand contact maps further clarified how each ligand interacted with the TRF1-TRFH domain over time (Figure 5.5B). These contact maps

included hydrogen bonds, hydrophobic contacts, ionic interactions, water bridges and halogen bonds. The upper panel of the contact map shows the total number of residue contacts made with the ligand over the trajectory, whereas the lower panel shows the frame wise distribution of these interactions. Residues capable of making more than one contact type are represented by more than one colour shade. This analysis is useful because it does not only show whether a ligand stayed near the binding site, but also which residues contributed most strongly to that retention during the simulation. For the A822 complex, the most frequent contacts were observed with ALA_133, SER_95, PHE_98, PHE_142 and ALA_134 (Figure 5.5B). Additional but weaker contacts were observed with PHE_90, ARG_102 and ALA_140. This interaction profile suggests that the parent scaffold remained associated with residues located mainly around the 95-98 and 133-142 regions of the binding site. Although these contacts were frequent, the ligand RMSD values showed that they did not keep A822 in one stable pose throughout the trajectory. The contact map therefore supports a model in which A822 remained engaged with the protein surface, but the engagement was less tightly anchored than in the active derivatives. However, the number of different positions may indicate that the overall population of the binding states of A822 is high (and higher compared to three studied derivatives) and it is consistent with the data presented in SPR experiments indicating stronger binding affinity of this lead molecule.

TRFiN41 showed a more concentrated contact pattern. The most dominant interaction was with THR_130, followed by PHE_142, ARG_102, ILE_109 and GLN_141 (Figure 5.5B). THR_130 alone contributed 5701 interaction events, which was the highest single residue contact count among all systems. This strong and persistent interaction is important because it provides a clear structural explanation for the low ligand RMSD observed for TRFiN41. Additional contacts with ARG_131, GLN_127, ILE_123, GLU_143 and GLU_106 further supported this binding mode. Compared with A822, TRFiN41 shifted the major interaction pattern toward the central binding region and showed a more focused contact network. The contact map therefore agrees with the view that TRFiN41 adopted a more anchored pose in the TRF1 site. TRFiN49 showed a broader interaction pattern than TRFiN41. The most frequent contacts were with ALA_105, ILE_123, GLU_106, GLN_127, LEU_86, PHE_98 and ILE_109, with additional contributions from LEU_79, CYS_126 and LEU_82 (Figure 5.5B). This distribution suggests that TRFiN49 did not rely on one dominant anchoring residue in the same way as TRFiN41. Instead, it was stabilised by a wider residue network spread across the binding region. This broader interaction pattern fits well with its ligand RMSD profile, which was higher than that of TRFiN41 but still far lower than that of A822. The TRFiN49 complex therefore appears to represent a binding mode in which the ligand remains well engaged with the site through multiple contacts rather than through one highly dominant contact. TRFiN72 also showed a strong and persistent interaction network, with major contacts involving GLU_106, PHE_142, ALA_105, ILE_109, CYS_126, ILE_123, ARG_102, THR_130 and ARG_131 (Figure 5.5B). Compared with A822, the dominant interaction pattern again shifted toward residues in the 102-130 region together with PHE_142. This distribution overlaps partly with both TRFiN41 and TRFiN49, which suggests that the derivatives share a related binding region but differ in the way they distribute contacts within that region. The RMSD values of TRFiN72 indicate that these contacts provided good site occupancy while still allowing moderate local movement. The contact map analysis therefore shows a clear difference between the parent scaffold and the derivatives. A822 interacted mainly with SER_95, PHE_98, ALA_133 and ALA_134, whereas TRFiN41, TRFiN49 and TRFiN72 showed stronger engagement with residues such as ARG_102, ALA_105, GLU_106, ILE_109, ILE_123, CYS_126, GLN_127, THR_130, ARG_131 and PHE_142 (Figure 5.5B). This shift is important because it suggests that chemical modification of the parent scaffold altered how the ligands were positioned against the TRF1-TRFH domain. The derivatives were not simply stronger versions of A822 in the same pose, but rather formed a different and more persistent interaction pattern within the protein surface.

Table 5.2. MM/GBSA binding energy summary for A822 and selected derivatives.

Energy parameters	A822	TRFiN41	TRFiN49	TRFiN72
Average Binding Energy (kcal/mol)	-58.28 $\pm$ 5.28	-45.71 $\pm$ 5.30	-60.64 $\pm$ 8.89	-56.93 $\pm$ 6.64
Coulomb	-25.46 $\pm$ 6.73	-14.97 $\pm$ 3.08	-4.68 $\pm$ 5.28	-10.03 $\pm$ 4.11
vdW	-44.00 $\pm$ 5.53	-39.33 $\pm$ 3.86	-50.40 $\pm$ 5.75	-49.52 $\pm$ 4.48
Lipo	-17.31 $\pm$ 3.77	-11.24 $\pm$ 1.71	-19.99 $\pm$ 2.68	-19.85 $\pm$ 2.45
Hbond	-0.46 $\pm$ 0.37	-0.71 $\pm$ 0.22	-0.19 $\pm$ 0.30	-0.38 $\pm$ 0.39
Packing	-1.82 $\pm$ 1.60	-6.50 $\pm$ 1.40	-2.51 $\pm$ 1.31	-1.66 $\pm$ 1.22
Solv GB	27.84 $\pm$ 3.93	24.87 $\pm$ 3.56	15.13 $\pm$ 3.54	22.35 $\pm$ 3.41

Note: Coulomb - Coulomb energy, vdW - Van der Waals energy, Lipo - Lipophilic energy, Hbond - Hydrogen bonding correction, Packing - pi-pi packing correction, Solv GB - Generalized Born electrostatic solvation energy.

The MM/GBSA calculations provided an energetic comparison of the ligand bound systems (Figure 5.5C; Table 5.2). Because of methodological restrictions, the absolute values of MM/GBSA energy are too low to be considered as physiologically relevant, but the relative differences can be discussed and are informative for our purpose as similar methodological approach has been applied. The average binding free energy was most favourable for TRFiN49, with a value of -60.64 ± 8.89 kcal/mol. A822 showed -58.28 ± 5.28 kcal/mol, while TRFiN72 showed -56.93 ± 6.64 kcal/mol. TRFiN41 gave the least favourable average binding free energy at -45.71 ± 5.30 kcal/mol. These values place TRFiN49 as the strongest binder by average calculated free energy, followed by A822 and TRFiN72, while TRFiN41 was weaker by this criterion despite showing the lowest ligand RMSD. TRFiN49 showed the most favourable van der Waals contribution (-50.40 ± 5.75 kcal/mol) and the strongest lipophilic term (-19.99 ± 2.68 kcal/mol), together with the lowest solvation penalty (15.13 ± 3.54 kcal/mol). This pattern suggests that the binding of TRFiN49 was strongly supported by efficient nonpolar packing and favourable burial within the site. TRFiN72 showed a similar profile, with strong van der Waals (-49.52 ± 4.48 kcal/mol) and lipophilic (-19.85 ± 2.45 kcal/mol) contributions, although the solvation penalty was higher than for TRFiN49. A822, in contrast, showed the strongest Coulomb term (-25.46 ± 6.73 kcal/mol), but this advantage was offset by the highest solvation penalty (27.84 ± 3.93 kcal/mol). This is consistent with the RMSD data, where the ligand remained associated with the protein but was not held in one well defined pose. TRFiN41 showed a different balance. Although it had the lowest ligand RMSD and the strongest local anchoring around THR_130, its Coulomb, van der Waals and lipophilic contributions were weaker than those of TRFiN49 and TRFiN72. The more favourable hydrogen bond correction and packing term of TRFiN41 did not compensate for the reduced nonbonded interaction energy. This explains why TRFiN41 can appear highly stable by RMSD but still shows a less favourable average MM/GBSA value. In this case, positional stability and calculated binding energy do not follow the same order. The data suggest that TRFiN41 retained a stable pose in the pocket, while TRFiN49 gained a stronger average interaction energy from broader hydrophobic and van der Waals contacts.

To examine how ligand binding affected protein motion beyond local contacts, PCA was carried out for the apo TRF1-TRFH domain and the ligand bound systems, and the trajectories were projected onto the first two principal components (Figure 5.6A). In the apo form, the protein showed a relatively dispersed distribution in the PC1-PC2 space. Principal component 1 (PC1) is the direction of collective atomic motion that accounts for the largest variation in the aligned MD trajectory and Principal component 2 (PC2) represents the second largest collective motion in the trajectory. This indicates that, in the absence of ligand, the TRF1-TRFH domain sampled a broader conformational space during the simulation. Such behaviour is expected for an unbound system, where the protein is free to move between several conformational states without restraint from ligand binding. The PCA plot of the A822 complex showed a more compact cluster of points than the apo system (Figure 5.6A). This suggests that the parent scaffold restricted the overall conformational spread of the protein, even though the ligand itself showed substantial movement by RMSD. The A822 complex therefore seems to represent a case where ligand binding reduced the amplitude of the sampled protein motions but

did not produce a strongly fixed ligand orientation. TRFiN41 showed a shifted conformational distribution compared with the apo form, but the spread of points remained at a similar level. This suggests that TRFiN41 changed the dominant collective motions of the protein while preserving a comparable degree of conformational flexibility. TRFiN72 showed broader scattering than the apo protein, which suggests that this ligand allowed the protein to explore a wider conformational space. TRFiN49 also showed a shifted scattering pattern relative to the apo system, indicating that ligand binding altered the dominant motions of the protein and changed the conformational states sampled during the trajectory. The FEL based on PC1 and PC2 provided a thermodynamic view of these conformational states (Figure 5.6B). The apo TRF1-TRFH domain showed two major minima. The global minimum was located at PC1 = 13.97 and PC2 = 3.19 with a free energy of -5.0 kcal/mol, while a second minimum was observed at PC1 = -32.98 and PC2 = 8.79 with an energy difference of 0.65 kcal/mol. This profile shows that the unbound protein moved between two major metastable states during the simulation.

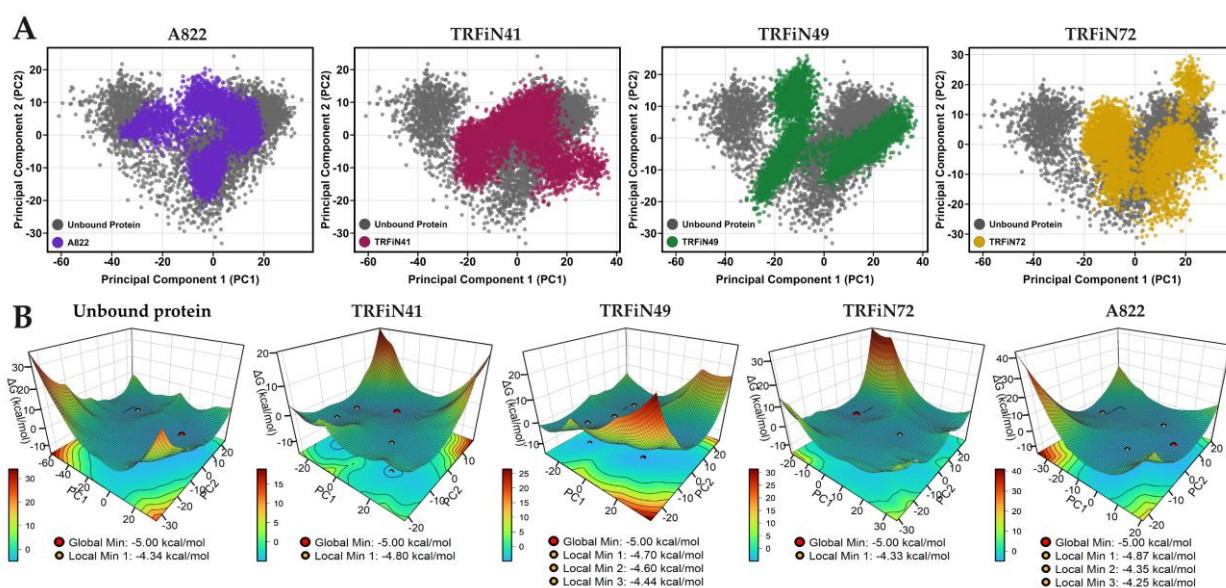


Figure 5.6 Principal component and free energy landscape analysis of the TRF1-TRFH domain in the apo form and in complex with A822, TRFiN41, TRFiN49 and TRFiN72 after 1000 ns molecular dynamics simulation. (A) PCA scatter plots showing the conformational space sampled by the apo protein and ligand bound systems along principal components 1 and 2 (PC1 and PC2). (B) Free energy landscapes constructed using PC1 and PC2 as reaction coordinates. The coloured surface represents the relative free energy distribution, and the marked points indicate the global and local minima identified for each system.

The A822 complex showed four minima in the FEL (Figure 5.6B). The global minimum was located at PC1 = 12.21 and PC2 = 1.59 with a free energy of -5.0 kcal/mol. Additional minima were located at PC1 = -0.44 and PC2 = -8.88, PC1 = 2.37 and PC2 = 8.93, and PC1 = -28.57 and PC2 = 2.65, with energy differences of 0.12, 0.61 and 0.75 kcal/mol. These closely spaced minima suggest that although A822 reduced the overall spread of protein conformations in PCA, the system still accessed several nearby metastable states. This fits the RMSD behaviour of the parent scaffold, where the ligand moved substantially within a protein framework that remained structurally stable. TRFiN41 also showed four minima in the FEL (Figure 5.6B). The global minimum was located at PC1 = 0.93 and PC2 = 4.85, and additional minima were found at PC1 = -17.85 and PC2 = -0.98, PC1 = -17.85 and PC2 = -9.75, and PC1 = 18.15 and PC2 = -9.75, with energy differences of 0.24, 0.69 and 0.88 kcal/mol. This pattern is consistent with the PCA result, where the system sampled a shifted but comparably broad conformational space relative to the apo protein. In the TRFiN41 complex, a stable ligand pose was therefore accompanied by access to several low energy protein states. TRFiN49 showed three minima in the FEL (Figure 5.6B), with the global minimum at PC1 = 12.28 and PC2 = -4.66 and two additional minima at PC1 = -12.99 and PC2 = 13.25 and at PC1 = -18.05 and PC2 = -8.50. The energy differences from the global minimum were 0.28 and 0.40 kcal/mol. This profile places TRFiN49 between the more complex four basin landscapes of A822 and TRFiN41 and the simpler two basin pattern of the apo and

TRFiN72 systems. The TRFiN49 complex therefore sampled more than one favourable conformational state but within a more limited basin structure than A822 or TRFiN41. TRFiN72 showed two major minima in the FEL (Figure 5.6B), with a global minimum at PC1 = -10.69 and PC2 = 2.96 and a second minimum at PC1 = 15.17 and PC2 = 0.004, separated by an energy difference of 0.62 kcal/mol. Although the PCA plot showed broader scattering for TRFiN72 than for the apo system, the FEL suggests that this motion occurred mainly within two dominant low energy basins rather than across many distinct metastable states. This is in line with its intermediate ligand RMSD and its persistent contact pattern involving GLU_106, PHE_142, ALA_105, ILE_109, CYS_126, ILE_123, ARG_102, THR_130 and ARG_131. TRFiN72 therefore combined moderate ligand stability, a strong residue contact network and a simpler free energy surface than A822 or TRFiN41.

5.6 Effects of TRFiN compounds and 4OHT on morphology, cell growth, motility, and cell death in MCF7 and HeLa cells

Live cell imaging and CellTraxx based tracking showed that treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT altered the behaviour of both MCF7 and HeLa cells in a clear and time dependent manner. The control cells in both models retained active growth and motility, whereas treated cells showed reduced expansion, restricted motility, enlarged morphology, and progressive accumulation of cell death. Although all compounds were active to some extent, the magnitude and pattern of response differed between compounds and between the two cell lines, indicating that the biological effect was influenced by cellular background. Morphological observation after 72 h of incubation showed a clear difference between control and treated cells in both models. In MCF7 cells, the untreated control retained the expected epithelial appearance, with dense colony like growth and close cell cell arrangement (Figure 5.7A). In contrast, cells treated with TRFiN41, TRFiN49, TRFiN72, or 4OHT were visibly enlarged and more widely separated. The treated populations no longer showed the compact organisation seen in the control. A similar change was also observed in HeLa cells (Figure 5.7D). The untreated HeLa control showed the expected adherent morphology, whereas all treated groups contained enlarged cells with altered appearance. This morphological change was common to all treatments in both cell lines and therefore appears to represent a shared response to compound exposure under the present conditions. Since enlargement was already visible at 72 h, before the highest levels of cell death were reached, this change is likely to represent an earlier phase of treatment response rather than only a terminal degenerative event.

The growth curves further supported these observations. In MCF7 cells, the control condition showed a steady increase in normalised growth across the 72 h observation period (Figure 5.7B). This is in agreement with the dense morphology seen in the control images. All treated groups showed a reduction in growth relative to the control, but the extent of inhibition differed between compounds. TRFiN41 produced strong suppression of growth in MCF7 cells and remained among the most active treatments throughout the recording period. 4OHT produced a very similar inhibitory pattern. TRFiN49 also suppressed MCF7 growth clearly, although its curve remained slightly above that of TRFiN41 and 4OHT. TRFiN72 showed the weakest effect in this cell line. Its curve remained closer to the control for a longer period and separated more clearly only at later time points. This suggests that TRFiN72 acted more slowly or less strongly on MCF7 growth dynamics than the other tested compounds. Thus, in MCF7 cells, TRFiN41 and 4OHT showed the strongest growth suppression, TRFiN49 showed intermediate activity, and TRFiN72 showed the weakest effect. A related pattern was seen in HeLa cells, although the ranking of the compounds was not identical (Figure 5.7E). The untreated HeLa control showed steady progressive growth along the observation period, indicating continued expansion of the culture. All treated HeLa groups showed reduced growth relative to control. TRFiN41 produced the strongest suppression of HeLa growth and caused an early flattening of the curve, indicating that expansion was strongly restricted soon after treatment. TRFiN72 also showed marked inhibition in HeLa cells and remained more active than TRFiN49 and A822. TRFiN49 reduced growth, but less strongly than TRFiN41. The effect of 4OHT was intermediate.

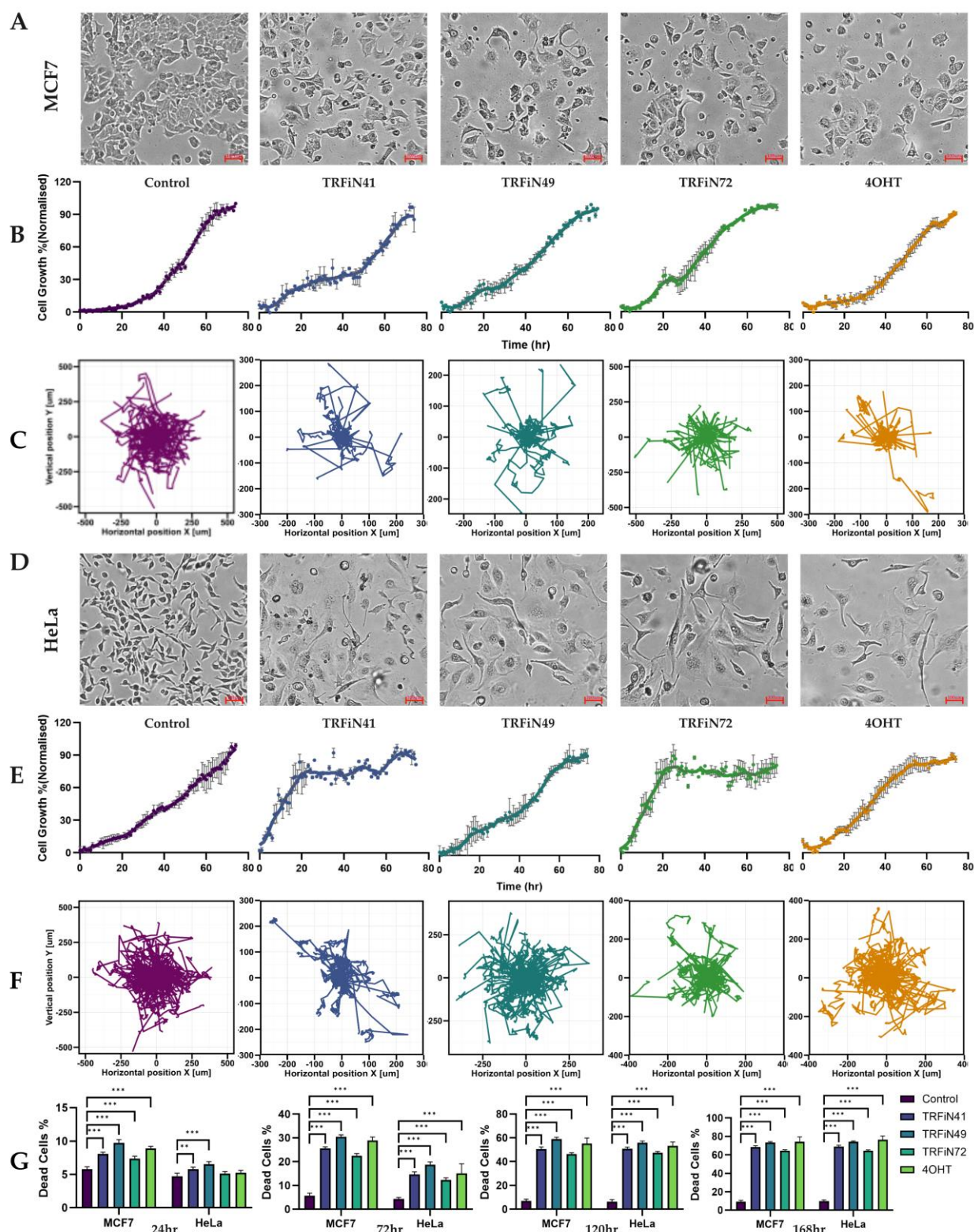


Figure 5.7. Effects of TRFiN compounds and 4OHT on morphology, cell growth, motility, and cell death in MCF7 and HeLa cells. (A,D) Representative morphology of MCF7 and HeLa cells, respectively, after 72 h of treatment under control, TRFiN41, TRFiN49, TRFiN72, and 4OHT conditions. (B,E) Normalised live cell growth curves of MCF7 and HeLa cells, respectively. (C,F) CellTraxx trajectory plots showing migration behaviour of MCF7 and HeLa cells, respectively. (G) Percentage of dead cells in MCF7 and HeLa cells at 24, 72, 120, and 168 h after treatment. The statistical analysis was conducted by two way ANOVA followed by Dunnett's post hoc test. Data are presented as mean $\pm$ SD ($n = 3$).

These data indicate that TRFiN41 was the most effective inhibitor of live cell expansion in HeLa cells under the present conditions. Therefore, while MCF7 cells responded strongly to both TRFiN41 and 4OHT, HeLa cells appeared especially sensitive to TRFiN41. The growth data indicate that the treatment effect was not simply a reduction in final cell number, but rather a change in the rate and pattern of cell expansion during continuous observation. In several treated groups, especially MCF7 cells treated with TRFiN41 or 4OHT and HeLa cells treated with TRFiN41, the curves rose during the early phase and then approached a plateau. This suggests that the cells remained present in the imaging field but lost the capacity for sustained growth seen in the controls. This interpretation fits well with the enlarged morphology observed at 72 h. The cells were still detectable, but their proliferative behaviour had clearly changed.

CellTraxx analysis showed that these compounds also altered single cell movement (Figure 5.7C,F). In MCF7 cells, the control tracks formed a wide and dense pattern around the origin, reflecting active movement during the recording period (Figure 5.7C). The treated groups showed visibly more restricted tracks. TRFiN41 reduced the overall spread of the trajectories, indicating suppression of motility. TRFiN49 and 4OHT produced the most confined tracking patterns in MCF7 cells, with marked restriction of movement away from the origin. TRFiN72 also reduced motility, although the extent of confinement was less pronounced than with TRFiN49 or 4OHT. Quantitative analysis supported this visual pattern. Control MCF7 cells showed the highest values for velocity, cumulative path length, and net displacement, while treated cells showed clear reductions in all of these parameters. The strongest suppression of MCF7 motility was observed with TRFiN49 and 4OHT, followed by TRFiN41, whereas TRFiN72 showed weaker but still clear inhibition. The HeLa CellTraxx data showed the same general tendency but with a somewhat different ranking of the compounds (Figure 5.7F). Control HeLa cells displayed broad and dense movement tracks, consistent with active migration. Treatment reduced this movement in all groups. TRFiN41 showed the strongest restriction of trajectory spread in HeLa cells and produced the clearest reduction in velocity and net displacement. TRFiN72 also suppressed motility, although less strongly than TRFiN41. TRFiN49 showed a measurable but weaker inhibitory effect, while 4OHT had the least pronounced effect in the HeLa tracking dataset. Therefore, in HeLa cells the TRFiN derivatives, especially TRFiN41, were more effective in reducing motility than the reference drug 4OHT. This is an important point, because it shows that the response to the compounds was not identical in the two cell lines. In MCF7 cells, TRFiN49 and 4OHT produced the strongest movement restriction, whereas in HeLa cells TRFiN41 showed the clearest effect. The motility and growth results complement each other closely. In both cell lines, the control cells showed active spreading, continuous increase in growth area, and broad motility tracks. After treatment, the cells did not only expand less, but also moved less. This indicates that the compounds affected dynamic cell behaviour at more than one level. The enlarged morphology seen at 72 h fits with this pattern. The cells appear to enter an altered phenotypic state characterised by reduced expansion, restricted movement, and visible enlargement. Such a phenotype suggests that the early response to treatment is mainly growth suppressive and behaviour altering rather than immediately destructive.

This is further supported by the dead cell analysis performed at 24, 72, 120, and 168 h (Figure 5.7G). In both cell lines, the untreated controls showed only low levels of dead cells at all time points. In contrast, all treated groups showed a progressive increase in dead cell percentage with increasing duration of exposure. At 24 h, the increase in cell death was still modest in both MCF7 and HeLa cells. This indicates that acute early toxicity was limited and that the initial drug response did not involve immediate large scale cell loss. At 72 h, the dead cell fraction had increased clearly above control in all treated groups. The increase was more pronounced in MCF7 cells than in HeLa cells, showing that MCF7 entered the death response earlier. In both lines, TRFiN49 and 4OHT produced the highest dead cell percentages at this stage, while TRFiN72 showed the lowest values among the active treatments. At 120 h, the percentage of dead cells increased strongly in all treated groups in both cell lines. The same trend continued at 168 h, where all active treatments produced extensive cell death.

In MCF7 cells, TRFiN49 remained the strongest TRFiN derivative and approached the effect of 4OHT closely. TRFiN41 also produced a strong late cytotoxic effect, whereas TRFiN72 remained the weakest of the three derivatives. In HeLa cells, the same general ranking was seen at the later time points, although the response developed more slowly than in MCF7. These findings show that the compounds induced progressive cell death during prolonged treatment, but that this occurred after the earlier changes in morphology, growth, and motility had already become evident.

This temporal sequence is important for interpretation of the overall response. At 72 h, treated cells were enlarged, growth was already strongly restricted, and migration was reduced, yet the dead cell fraction had not yet reached the very high levels seen at 120 h and 168 h. This suggests that the first response to treatment was a change in cell state, characterised by reduced proliferative behaviour and lower motility. Cell death accumulated later as exposure continued. The data therefore support a model in which the compounds first impose a cytostatic like or stress associated effect, followed by a progressive cytotoxic outcome during prolonged incubation. The compound specific patterns also help distinguish the behaviour of the three TRFiN derivatives. TRFiN41 was the most consistent inhibitor across the live cell datasets. It strongly suppressed HeLa growth, markedly reduced HeLa motility, and also showed strong activity in MCF7 growth suppression. Its effect on cell death was also substantial at the later time points, although it remained slightly below TRFiN49 and 4OHT in the final dead cell percentages. This suggests that TRFiN41 may exert a strong early effect on cell behaviour and proliferation, with subsequent development of marked cytotoxicity during longer exposure. TRFiN49 showed a somewhat different response profile. In the growth and tracking experiments, it was especially active in MCF7 cells, where it strongly reduced motility and clearly suppressed expansion. In the dead cell assay, TRFiN49 produced the highest values among the TRFiN derivatives in both MCF7 and HeLa cells at the later time points. This indicates that TRFiN49 may be particularly effective in driving cells toward death during prolonged treatment. Its activity approached that of 4OHT closely, especially in MCF7 cells. TRFiN72 was active in all assays, but its effect was weaker than that of TRFiN41 and TRFiN49. It reduced growth and motility and increased the dead cell fraction, but the magnitude of response was generally lower, particularly in MCF7 growth dynamics and in the later cell death data. The comparison with 4OHT places these findings in a useful biological context. In MCF7 cells, 4OHT strongly suppressed growth and motility and induced high late stage cell death. TRFiN41 matched 4OHT closely in growth inhibition, while TRFiN49 approached it most closely in the dead cell assay. In HeLa cells, 4OHT was less effective than TRFiN41 in the motility dataset but remained a strong inducer of late cell death. Thus, the best TRFiN derivatives showed activity that was comparable to the reference drug in several of the measured phenotypic readouts. The results indicate that treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT caused a linked sequence of responses in both MCF7 and HeLa cells. The earliest visible features were enlargement of the cells, reduced expansion of the culture, and reduced motility. With longer incubation, these changes were followed by marked accumulation of dead cells. Within this set of compounds, TRFiN41 emerged as the most robust inhibitor of live cell growth behaviour, especially in HeLa cells, whereas TRFiN49 showed the strongest long term cytotoxic effect among the TRFiN derivatives and came closest to 4OHT. TRFiN72 remained active but less potent than the other two lead compounds.

5.7 Cell cycle redistribution and replication fork progression after treatment with TRFiN compounds

Cell cycle analysis was performed using combined PI and BrdU staining. This approach allowed DNA content and ongoing DNA synthesis to be read together and gave a more accurate view of the events occurring during cell cycle progression than PI alone. PI defined the total DNA content, while BrdU identified cells that were actively synthesising DNA. This was important in the present study because some treatments did not simply shift cells between G0/G1 and G2/M, but also changed the fraction of cells that remained in S phase. Representative cell cycle profiles and quantified G0/G1, S phase, and G2/M fractions are presented in Figure 5.8A-F. Representative PI/BrdU plots at 168 h, confocal images of labelled DNA fibres, and quantification of the IdU/CldU ratio are presented in

Figure 5.9A-D. The cell cycle response differed between MCF7 and HeLa cells and changed considerably with the duration of treatment.

In MCF7 cells, the earliest clear redistribution was observed after treatment with TRFiN72 and 4OHT. At 24 h, the control population contained 58.05% of cells in G0/G1 and 5.83% in S phase. TRFiN72 increased the G0/G1 fraction to 64.10% and reduced the S phase fraction to 1.68%, while 4OHT increased G0/G1 to 65.00% and reduced S phase to 1.73% (Figure 5.8B,E). These changes corresponded to an approximately 70-71% reduction in the S phase fraction and a 10-12% increase in G0/G1 relative to the control. TRFiN41 and TRFiN49 produced smaller changes at this early time point. TRFiN41 gave a G0/G1 fraction of 55.95% and an S phase fraction of 7.45%, whereas TRFiN49 gave 54.45% in G0/G1 and 5.11% in S phase. The initial response in MCF7 cells was therefore most evident under TRFiN72 and 4OHT. The redistribution became more evident after 72 h of treatment. The G0/G1 fraction in the control decreased to 46.10%, while the S phase fraction increased to 9.72%. TRFiN41 and TRFiN72 increased the G0/G1 population to approximately 60% and reduced the S phase fraction to 5.70% and 5.24%, respectively. These changes represented an approximately 30% increase in G0/G1 and a 41-46% reduction in S phase relative to the control. Treatment with 4OHT produced a similar distribution, with 58.60% of cells in G0/G1 and 6.02% in S phase. TRFiN49 caused a less pronounced change, increasing G0/G1 to 50.50% and reducing S phase to 7.46%. The G2/M fraction was also lower in each treated group than in the corresponding control at this time point. Thus, the 72 h profiles showed a clear reduction in the proportion of actively synthesising cells, particularly after treatment with TRFiN41, TRFiN72, and 4OHT.

A stronger G0/G1 enriched distribution was recorded in MCF7 cells after 120 h (Figure 5.8F). The control contained 41.20% of cells in G0/G1 and 16.47% in S phase. Treatment increased the G0/G1 fraction to 58.35-66.45%, corresponding to a 1.42- to 1.61-fold increase relative to the control. The highest G0/G1 fraction was observed with 4OHT, followed by TRFiN72, TRFiN41, and TRFiN49. At the same time, the S phase population decreased to between 4.29% and 8.06%. This represented a reduction of approximately 51-74% compared with the control. The largest reduction in S phase was recorded after 4OHT treatment, while TRFiN41 and TRFiN72 also produced clear decreases. The G0/G1 enriched distribution remained evident at 168 h. The control population contained 48.10% of cells in G0/G1, whereas the treated groups contained 70.85-78.20%. TRFiN41 produced the highest G0/G1 fraction, followed by TRFiN49, TRFiN72, and 4OHT. These values were approximately 1.47- to 1.63-fold higher than the control. The S phase fraction remained below 10% in all groups. TRFiN72 and 4OHT produced values slightly below the control, while TRFiN41 and TRFiN49 produced values slightly above the control. Despite these differences in the small S phase population, all four treatments maintained a markedly greater proportion of MCF7 cells in G0/G1 than the untreated control. The time course analysis therefore showed that the MCF7 response developed from a selective early effect under TRFiN72 and 4OHT into a broader G0/G1 enriched distribution under all treatments at the later time points.

HeLa cells showed a different early cell cycle pattern. At 24 h, only 0.63% of control cells were assigned to S phase, while 58.24% were in G0/G1 and 41.90% were in G2/M. Each treatment markedly increased the S phase fraction. TRFiN41 increased S phase to 14.50%, while TRFiN49 and TRFiN72 each increased it to 13.53%. The highest S phase fraction was observed with 4OHT at 24.70% (Figure 5.8A,C). These values represented approximately 21- to 39-fold increases relative to the control. The increase in the BrdU positive fraction was accompanied by changes in the other cell cycle populations. G0/G1 was reduced most clearly by TRFiN49 and 4OHT, while the G2/M fraction was lower under TRFiN41, TRFiN72, and 4OHT than in the control. The early HeLa profiles were therefore characterised by a marked increase in the proportion of cells assigned to S phase rather than by immediate G0/G1 enrichment. At 72 h, the HeLa response became more dependent on the individual treatment. The control contained 16.00% of cells in S phase. TRFiN41 increased this fraction to 36.74%, corresponding to a 2.30-fold increase, while 4OHT increased it to 22.85%, corresponding to a 1.43-fold increase. These increases were accompanied by reductions in the G2/M fraction. By comparison,

TRFiN49 reduced the S phase population to 1.26%, an approximately 92% reduction relative to control, while TRFiN72 reduced it to 7.54%, an approximately 53% reduction. The G0/G1 fractions under TRFiN49 and TRFiN72 remained close to or slightly above the control. Therefore, TRFiN41 and 4OHT maintained a large BrdU positive population at 72 h, whereas TRFiN49 and TRFiN72 produced a clear reduction in the S phase population.

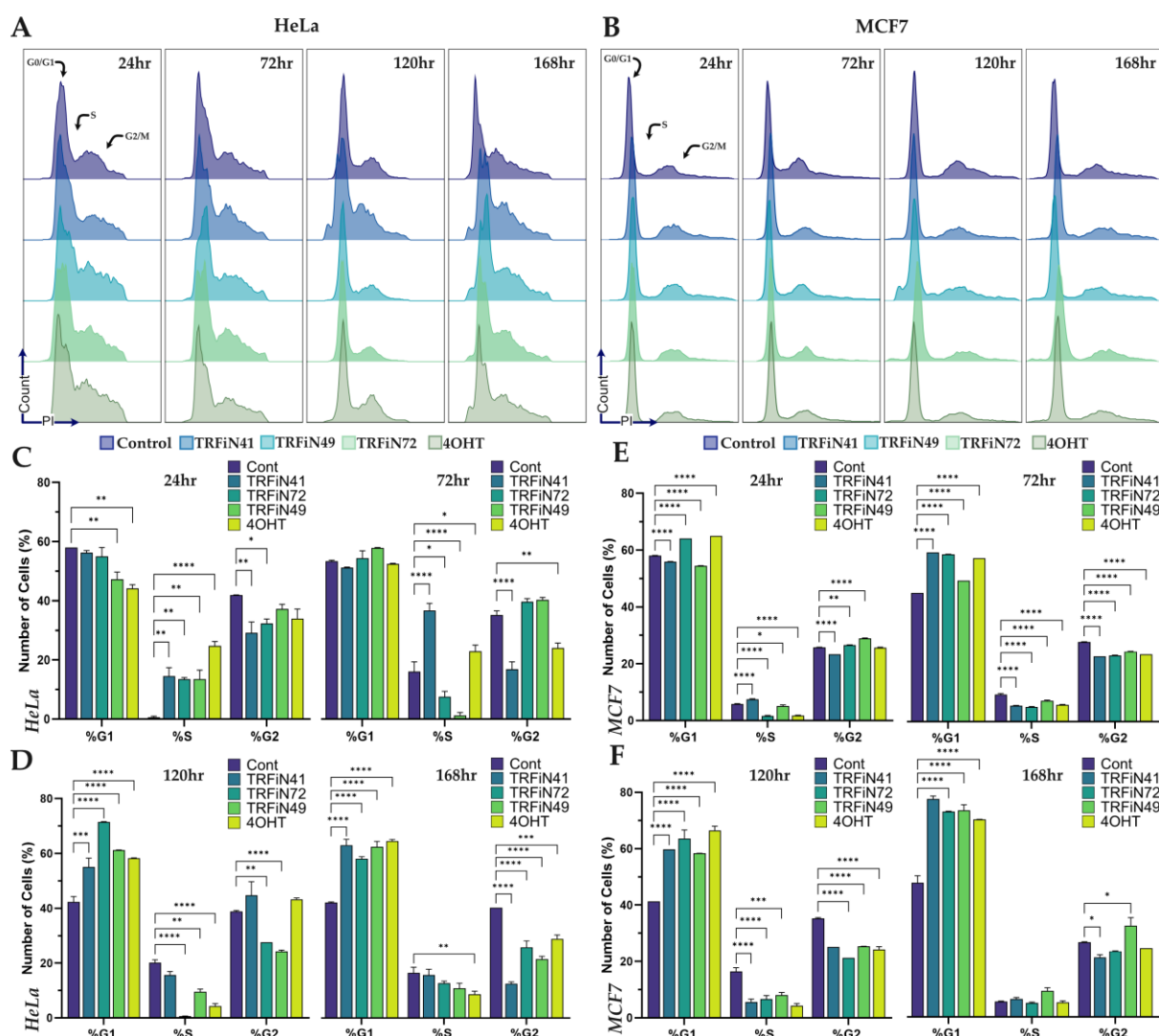


Figure 5.8. Effect of TRFiN compounds and 4OHT on cell cycle distribution in HeLa and MCF7 cells at different time points. (A,B) Representative PI histograms of HeLa and MCF7 cells, respectively, at 24, 72, 120, and 168 h under control, TRFiN41, TRFiN49, TRFiN72, and 4OHT conditions. (C,D) Quantified cell cycle fractions of HeLa cells at 24 and 72 h, and at 120 and 168 h, respectively. (E,F) Quantified cell cycle fractions of MCF7 cells at 24 and 72 h, and at 120 and 168 h, respectively. Cell cycle analysis was performed using combined PI and BrdU staining. The statistical analysis was conducted by two way ANOVA followed by Dunnett's post hoc test. Data are presented as mean $\pm$ SD (n = 3).

The HeLa cell cycle distribution changed further during prolonged treatment (Figure 5.8D). At 120 h, the control population contained 42.30% of cells in G0/G1 and 20.15% in S phase. All treatments increased the G0/G1 fraction, although the magnitude differed between compounds. TRFiN72 produced the clearest redistribution, increasing G0/G1 to 71.50% and reducing S phase to 0.63%. This represented an approximately 69% increase in G0/G1 and a 97% reduction in S phase relative to the control. TRFiN49 increased G0/G1 to 61.25% and reduced S phase to 9.50%, while 4OHT increased G0/G1 to 58.30% and reduced S phase to 4.25%. TRFiN41 produced a smaller G0/G1 increase and retained a larger S phase population than the other treatments, with 55.10% of cells in G0/G1 and 15.67% in S phase. By 168 h, all treated HeLa groups showed greater G0/G1 fractions than the control. The control contained 44.75% of cells in G0/G1, whereas the treated populations ranged from 61.70%

under TRFiN72 to 68.65% under 4OHT. TRFiN41 and TRFiN49 produced similar G0/G1 fractions of 67.00% and 66.30%, respectively. These changes corresponded to increases of approximately 38-53% relative to the control. The S phase fraction was lower than control under every treatment, although the magnitude of reduction differed. TRFiN41 produced only a small reduction, whereas TRFiN49, TRFiN72, and 4OHT reduced the S phase fraction by approximately 34%, 23%, and 47%, respectively. The G2/M fraction was also lower in each treated group than in the control. Thus, the early increase in the S phase population observed in HeLa cells was followed by a later distribution containing more cells in G0/G1 and fewer cells in S phase or G2/M.

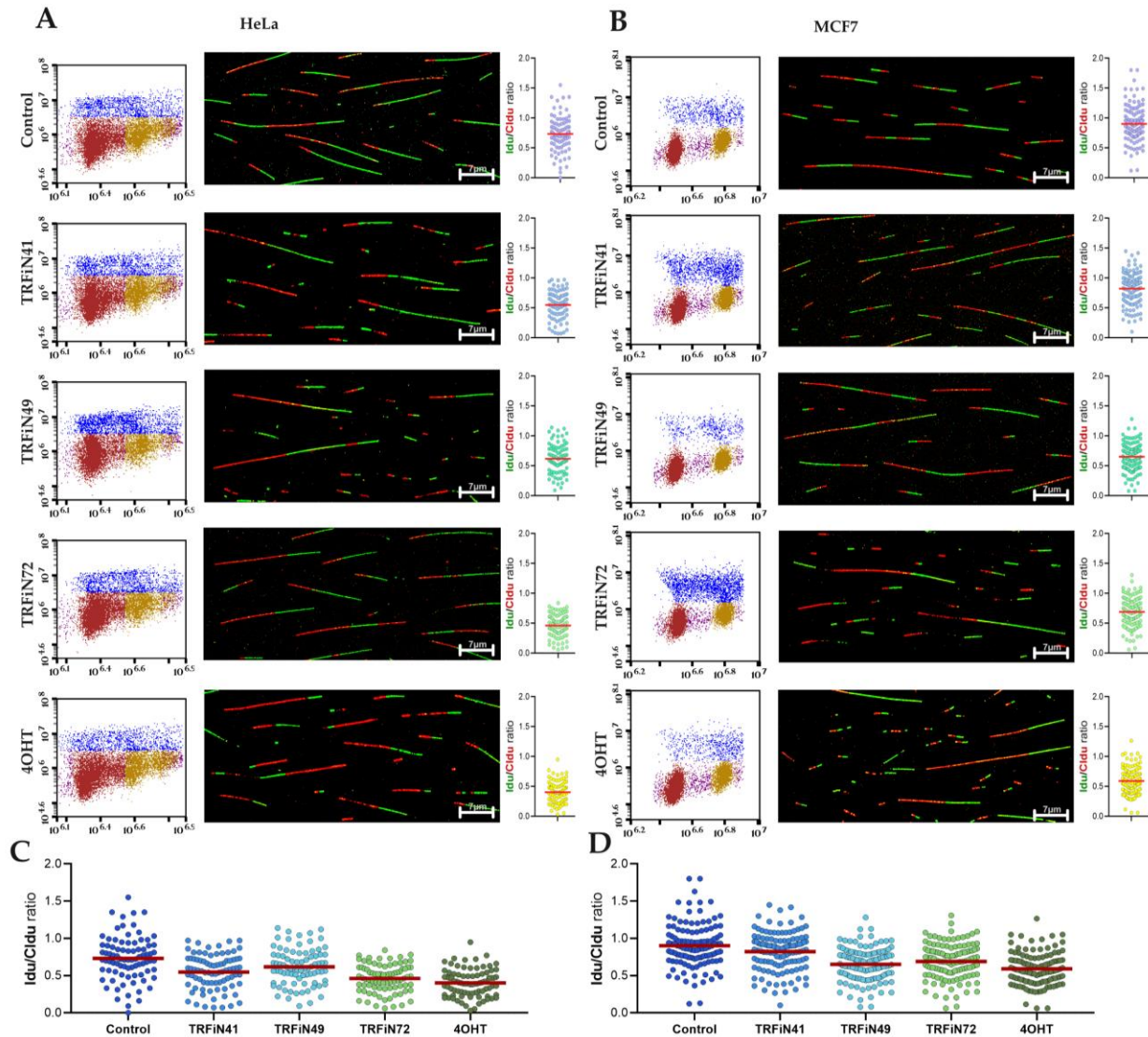


Figure 5.9. Effect of TRFiN compounds and 4OHT on PI/BrdU profile and replication fork progression in HeLa and MCF7 cells. (A,B) Representative PI/BrdU flow cytometric dot plots at 168 h and confocal images of DNA fibres in HeLa and MCF7 cells, respectively, under control, TRFiN41, TRFiN49, TRFiN72, and 4OHT conditions. (C,D) Quantification of the IdU/CldU ratio in HeLa and MCF7 cells, respectively. A reduced IdU/CldU ratio indicates impaired replication fork progression after treatment. The statistical analysis was conducted by two way ANOVA followed by Dunnett's post hoc test. Data are presented as mean $\pm$ SD ($n = 4$).

DNA fibre analysis was performed to examine treatment related changes in sequentially labelled replication tracks (Figure 5.9A-D). In HeLa cells, the mean IdU/CldU ratio was 0.7318 ± 0.1958 in the control. This ratio decreased to 0.5473 ± 0.1330 after TRFiN41 treatment, 0.6164 ± 0.2421 after TRFiN49 treatment, 0.4610 ± 0.1872 after TRFiN72 treatment, and 0.4008 ± 0.1311 after 4OHT treatment. Relative to the control, these values represented reductions of approximately 25%, 16%, 37%, and 45%, respectively. The largest decrease was therefore recorded under 4OHT, followed by TRFiN72 and TRFiN41. TRFiN49 produced a smaller but still measurable reduction. A similar decrease in the

measured IdU/CldU ratio was observed in MCF7 cells. The control ratio was 0.90 ± 0.20 and decreased to 0.82 ± 0.27 with TRFiN41, 0.65 ± 0.13 with TRFiN49, 0.69 ± 0.34 with TRFiN72, and 0.59 ± 0.12 with 4OHT. These values corresponded to reductions of approximately 9%, 28%, 23%, and 34%, respectively. The largest decrease was again recorded with 4OHT. Among the TRFiN compounds, TRFiN49 produced the greatest reduction in MCF7 cells, followed by TRFiN72, while TRFiN41 produced a smaller change. The representative DNA fibre images showed visible differences in the relative lengths of the sequentially labelled tracks between the control and treated groups.

5.8 Time dependent induction of DNA damage in HeLa and MCF7 cells

The time dependent induction of DNA damage was examined by measuring the percentage of γ H2AX positive cells after 24, 72, 120, and 168 h of treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT. Representative γ H2AX flow cytometry histograms for HeLa and MCF7 cells are presented in Figure 5.10A and B, respectively, while the quantified γ H2AX positive populations are presented in Figure 5.10E and F. In untreated HeLa cells, the γ H2AX positive fraction remained relatively low during the first 120 h, increasing from $8.62 \pm 0.94\%$ at 24 h to $9.15 \pm 1.06\%$ at 72 h and $9.63 \pm 0.96\%$ at 120 h. A moderate increase to $13.18 \pm 1.96\%$ was recorded at 168 h. At 24 h, the earliest treatment related changes were observed with TRFiN41 and 4OHT. TRFiN41 increased the γ H2AX positive population to $11.97 \pm 1.61\%$, corresponding to a 1.39-fold increase over the control, while 4OHT increased it to $13.28 \pm 2.11\%$, corresponding to a 1.54-fold increase. TRFiN49 produced only a small change at this time point, with $9.45 \pm 1.22\%$ γ H2AX positive cells, whereas the value under TRFiN72 remained slightly below the control at $7.48 \pm 1.02\%$. These early differences are visible in the HeLa histograms in Figure 5.10A and in the quantified values in Figure 5.10E.

The treatment response in HeLa cells became more evident at 72 h. The γ H2AX positive fraction increased to $21.68 \pm 1.57\%$ under TRFiN41 and $23.72 \pm 3.14\%$ under 4OHT, representing 2.37- and 2.59-fold increases over the corresponding control. TRFiN49 increased the positive population to $17.24 \pm 1.62\%$, while TRFiN72 increased it to $14.26 \pm 3.78\%$. These values were 1.88- and 1.56-fold above the control, respectively. Thus, all treatments increased the γ H2AX positive fraction by 72 h, although the increase remained greater under TRFiN41 and 4OHT than under TRFiN49 and TRFiN72. The rightward shifts of the treated populations in Figure 5.10A followed the same distribution as the quantified measurements in Figure 5.10E. A greater separation between the HeLa treatment groups was observed at 120 h. TRFiN41 increased the γ H2AX positive population to $42.30 \pm 2.17\%$, which was 4.39-fold above the control and approximately twice the values measured under TRFiN49 and TRFiN72. The γ H2AX positive fractions under TRFiN49 and TRFiN72 were $18.22 \pm 2.37\%$ and $18.37 \pm 1.96\%$, respectively, corresponding to approximately 1.9-fold increases over the control. Treatment with 4OHT produced an intermediate response of $24.00 \pm 2.33\%$, which was 2.49-fold above the control. The difference between TRFiN41 and the other treatments at this time point is visible in Figure 5.10E, where the TRFiN41 treated population showed a greater increase between 72 and 120 h.

The γ H2AX positive fraction increased further in every treated HeLa group at 168 h. TRFiN41 produced the highest value at $65.17 \pm 4.15\%$, representing a 4.95-fold increase over the control. TRFiN49 produced the second highest value at $55.34 \pm 3.73\%$, corresponding to a 4.20-fold increase. Treatment with 4OHT increased the positive fraction to $51.27 \pm 4.07\%$, which was 3.89-fold above the control, while TRFiN72 increased it to $40.07 \pm 1.84\%$, corresponding to a 3.04-fold increase. The HeLa time course profiles in Figure 5.10A show that the separation between the untreated and treated populations became greater with increasing treatment duration. Among the tested compounds, TRFiN41 produced the largest increase at both 120 and 168 h. TRFiN49 showed a smaller response during the first 120 h but increased strongly between 120 and 168 h, reaching a late value close to that produced by 4OHT. TRFiN72 produced the lowest late γ H2AX value among the treated HeLa groups, although its positive fraction remained approximately threefold higher than the control.

MCF7 cells showed less change during the first 24 h and a greater response during prolonged treatment. The untreated population contained $4.97 \pm 0.72\%$ γ H2AX positive cells at 24 h, $7.59 \pm 1.02\%$ at 72 h, $10.53 \pm 2.33\%$ at 120 h, and $10.11 \pm 1.33\%$ at 168 h. At 24 h, the treated values remained within a narrow range of 5.06–5.92% and were close to the untreated control, as shown by the overlapping histogram distributions in Figure 5.10B and the quantified measurements in Figure 5.10F. TRFiN41 produced the highest value at this early time point, but the increase was limited to $5.92 \pm 0.78\%$. TRFiN49, TRFiN72, and 4OHT produced values of $5.25 \pm 0.92\%$, $5.06 \pm 0.82\%$, and $5.53 \pm 0.80\%$, respectively. These measurements show that none of the treatments produced a large increase in the γ H2AX positive MCF7 population during the first 24 h.

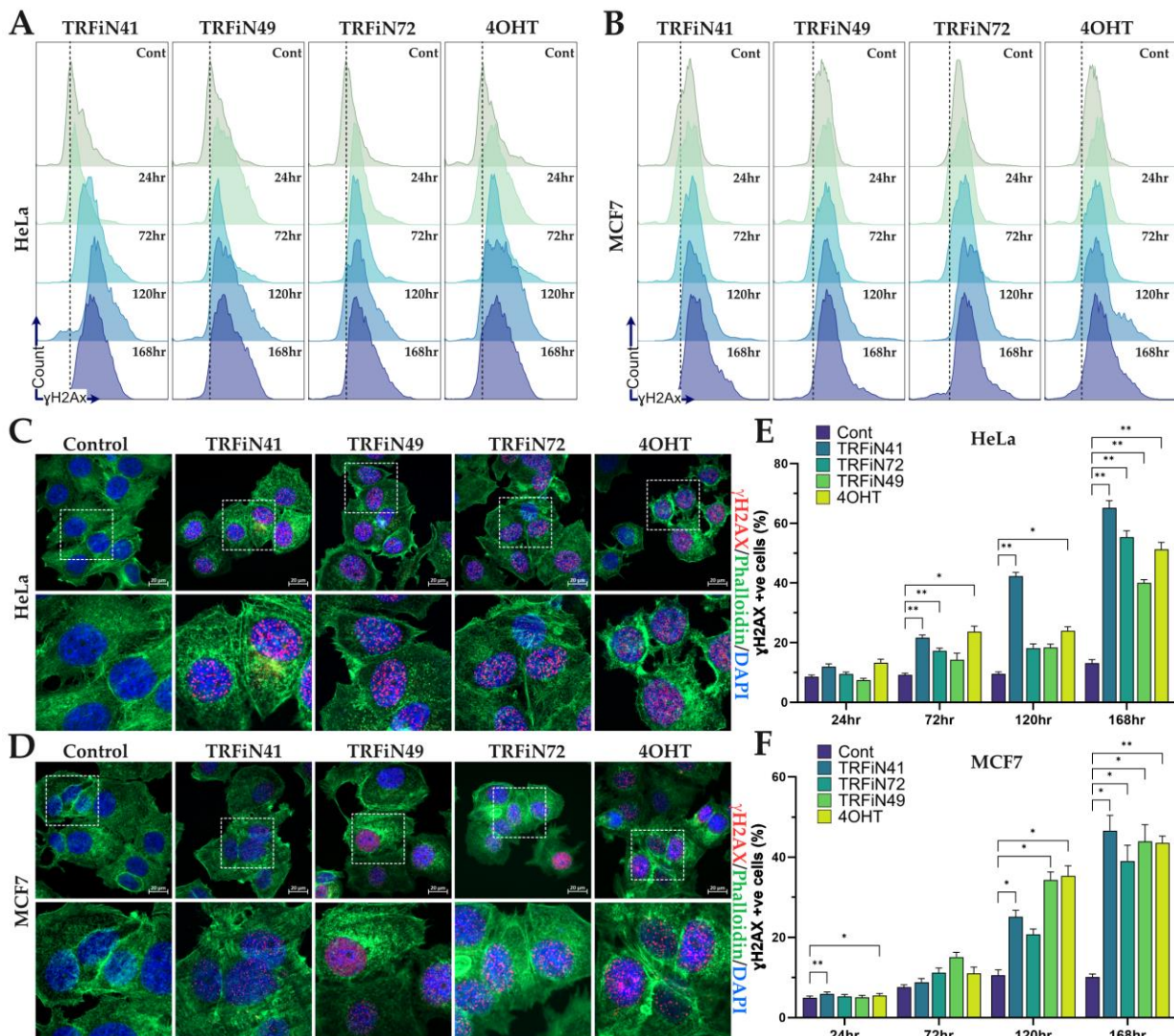


Figure 5.10. Effect of TRFiN compounds and 4OHT on DNA damage in HeLa and MCF7 cells. (A,B) Representative γ H2AX flow cytometry histograms of HeLa and MCF7 cells, respectively, at 24, 72, 120, and 168 h after treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT, with untreated cells as control. (C,D) Representative immunofluorescence images of γ H2AX, DAPI, and phalloidin in HeLa and MCF7 cells, respectively. (E,F) Quantification of γ H2AX positive cells in HeLa and MCF7 cells, respectively, at different time points after treatment. The statistical analysis was conducted by two way ANOVA followed by Dunnett's post hoc test. Data are presented as mean $\pm$ SD ($n = 3$).

The response in MCF7 cells became more apparent at 72 h, although the order of the treatment groups differed from that observed in HeLa cells. TRFiN72 produced the highest value at $15.09 \pm 2.06\%$, which was approximately twofold above the control. TRFiN49 and 4OHT increased the γ H2AX positive fractions to $11.19 \pm 2.03\%$ and $11.01 \pm 2.68\%$, corresponding to 1.47- and 1.45-fold increases, respectively. TRFiN41 produced a smaller increase to $8.81 \pm 1.60\%$, which was 1.16-fold above the control. The MCF7 histograms in Figure 5.10B show a greater shift under TRFiN72 than

under the other treatments at 72 h, and the same difference is present in the quantified values in Figure 5.10F. A larger increase was recorded in MCF7 cells at 120 h. TRFiN72 increased the γ H2AX positive fraction to $34.30 \pm 3.49\%$, while 4OHT produced a similar value of $35.38 \pm 4.28\%$. These values were 3.26- and 3.36-fold above the control, respectively. TRFiN41 increased the positive population to $25.10 \pm 2.88\%$, corresponding to a 2.38-fold increase, while TRFiN49 increased it to $20.77 \pm 2.28\%$, corresponding to a 1.97-fold increase. TRFiN72 and 4OHT therefore produced the highest intermediate responses in MCF7 cells, whereas TRFiN49 produced the lowest treated value at this time point. By 168 h, all four treatments produced a large γ H2AX positive population in MCF7 cells. TRFiN41 produced the highest mean value at $46.60 \pm 6.61\%$, representing a 4.61-fold increase over the control. TRFiN72 and 4OHT produced closely related values of $43.90 \pm 7.21\%$ and $43.57 \pm 2.91\%$, corresponding to 4.34- and 4.31-fold increases, respectively. TRFiN49 increased the positive fraction to $39.00 \pm 6.86\%$, which was 3.86-fold above the control. Although the variation between replicates was greater for some treatments at this time point, each treated population remained substantially higher than the untreated group in Figure 5.10F. The increase between 120 and 168 h was particularly large under TRFiN41 and TRFiN49, whereas TRFiN72 and 4OHT had already produced high γ H2AX positive fractions by 120 h.

Direct comparison of the two cell lines showed that the timing and relative strength of the γ H2AX response differed between treatments. At 72 h, TRFiN41 produced $21.68 \pm 1.57\%$ γ H2AX positive cells in HeLa cells compared with $8.81 \pm 1.60\%$ in MCF7 cells, giving an approximately 2.46-fold higher value in HeLa. At the same time point, 4OHT produced $23.72 \pm 3.14\%$ positive HeLa cells and $11.01 \pm 2.68\%$ positive MCF7 cells, corresponding to an approximately 2.15-fold difference. TRFiN49 also produced a higher value in HeLa cells at 72 h, whereas the response to TRFiN72 was similar in the two cell lines. At 120 h, TRFiN41 remained higher in HeLa cells, but TRFiN72 produced the opposite pattern. The γ H2AX positive fraction after TRFiN72 treatment reached $34.30 \pm 3.49\%$ in MCF7 cells compared with $18.37 \pm 1.96\%$ in HeLa cells, giving an approximately 1.87-fold higher value in MCF7. A similar, but smaller, difference was observed with 4OHT at 120 h. At 168 h, HeLa cells retained higher values under TRFiN41, TRFiN49, and 4OHT, while TRFiN72 produced similar late responses in the two cell lines. The immunofluorescence images provided a separate visual assessment of the γ H2AX response. Untreated HeLa cells in Figure 5.10C showed weak nuclear γ H2AX staining, whereas the treated cells showed stronger focal signals within the nuclei. The increase was most apparent in cells treated with TRFiN41, TRFiN49, and 4OHT. Untreated MCF7 cells in Figure 5.10D also showed weak γ H2AX staining, while treatment increased the number and intensity of nuclear γ H2AX positive regions. The stronger nuclear staining in the treated HeLa and MCF7 cells followed the increase in γ H2AX positive populations measured by flow cytometry.

5.9 Loss of mitochondrial membrane potential and mitochondrial structural damage

Mitochondrial changes after treatment were examined at both the functional and structural levels. Mitochondrial membrane potential was measured using the JC-1 assay. Mitochondrial organisation was examined by MitoTracker Green staining and confocal microscopy, and ultrastructural changes were analysed by TEM. In the JC-1 assay, the ratio between the red fluorescent polymer form and the green fluorescent monomer form was used as an indicator of mitochondrial membrane potential. A decrease in this ratio indicates mitochondrial depolarisation. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was used as a positive control for loss of membrane potential. In HeLa cells, the untreated control remained stable throughout the experiment, with a JC-1 polymer/monomer ratio of 1.00 ± 0.01 at all analysed time points.

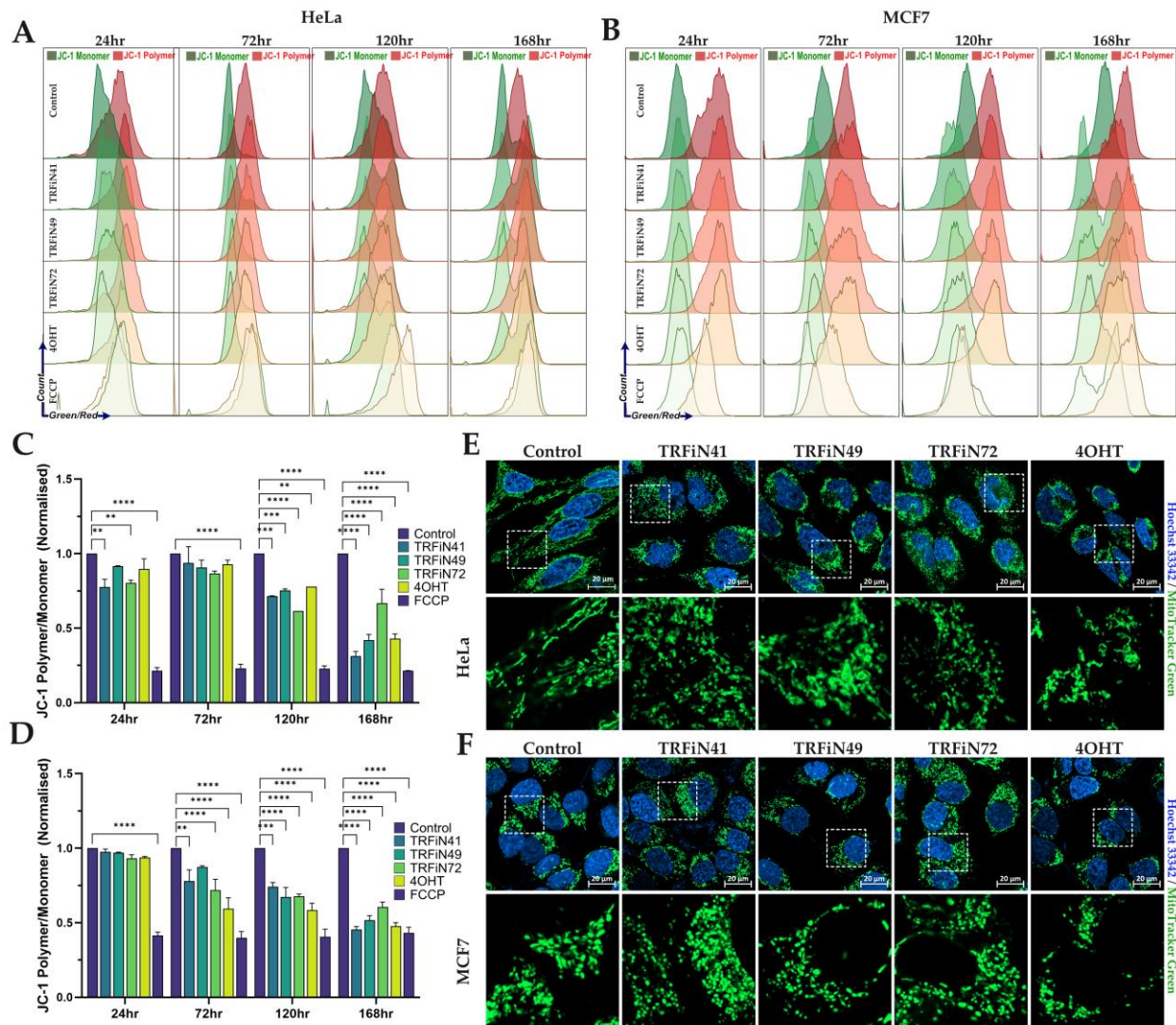


Figure 5.11. Effect of TRFiN compounds and 4OHT on mitochondrial membrane potential in HeLa and MCF7 cells. (A,B) Representative JC-1 monomer and polymer histograms of HeLa and MCF7 cells, respectively, at 24, 72, 120, and 168 h after treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT, with untreated cells as control. FCCP was used as a positive control. (C,D) Quantification of the JC-1 polymer/monomer ratio in HeLa and MCF7 cells, respectively, at different time points after treatment. (E,F) Representative confocal microscopic images of MitoTracker Green stained HeLa and MCF7 cells, respectively, after treatment. The statistical analysis was conducted by two way ANOVA and post hoc Dunnett's test. The data represent mean $\pm$ SD ($n = 3$), $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

JC-1 analysis showed a time dependent loss of mitochondrial membrane potential (Figure 5.11A–D). In HeLa cells, the polymer to monomer ratio fell by 8–22% at 24 h and by 24–38% at 120 h. By 168 h, the loss reached 68% with TRFiN41, 58% with TRFiN49, 57% with 4OHT, and 33% with TRFiN72. MCF7 cells responded more slowly: the reduction was no more than 6% at 24 h, increased to 12–40% at 72 h and 25–41% at 120 h, and reached 39–54% at 168 h. TRFiN41 and 4OHT produced the strongest late decreases in MCF7 cells. These changes show delayed mitochondrial depolarisation in both cell lines, with an earlier response in HeLa cells. The confocal microscopy images after MitoTracker Green staining showed that the functional change measured by JC-1 was accompanied by a marked change in mitochondrial organisation. In HeLa control cells, mitochondria formed an elongated and filament like network distributed through the cytoplasm. This pattern was lost after treatment, where the mitochondrial signal became fragmented, shortened, and clustered (Figure 5.11E). Under TRFiN41, the network appeared broken into short elements and small clusters. TRFiN49 also showed strong clustering with a discrete mitochondrial pattern. TRFiN72 produced a broken and shortened network, and 4OHT caused a sparse and strongly disrupted pattern in which most of the signal appeared as small discrete structures.

The enlarged fields in the lower panels of Figure 5.11E-F make this change clearer. A similar pattern was seen in MCF7 cells. Control cells showed a more continuous mitochondrial distribution, whereas the treated groups showed fragmentation and loss of the normal network pattern (Figure 5.11F). TRFiN41 and TRFiN49 produced fragmented and clustered mitochondria, TRFiN72 also disrupted the network, and 4OHT caused a strong breakdown of the mitochondrial pattern into shortened and discrete mitochondrial fragments. These images show that the decrease in mitochondrial membrane potential was accompanied by visible disruption of the mitochondrial network in both cell lines. Transmission electron microscopy showed direct mitochondrial ultrastructural damage in both HeLa and MCF7 cells. In untreated HeLa cells, mitochondria had elongated or oval profiles with preserved cristae and compact internal structure. After treatment, mitochondria appeared abnormal in shape and internal organisation (Figure 5.12A). In the TRFiN41 group, mitochondria were more rounded and swollen, with disturbed internal structure. TRFiN49 treated cells contained enlarged rounded mitochondria with less regular cristae. TRFiN72 treated cells also showed swollen mitochondria and altered internal organisation. In the 4OHT group, mitochondrial damage was also clear, with abnormal shape and disturbed internal structure in the enlarged TEM field.

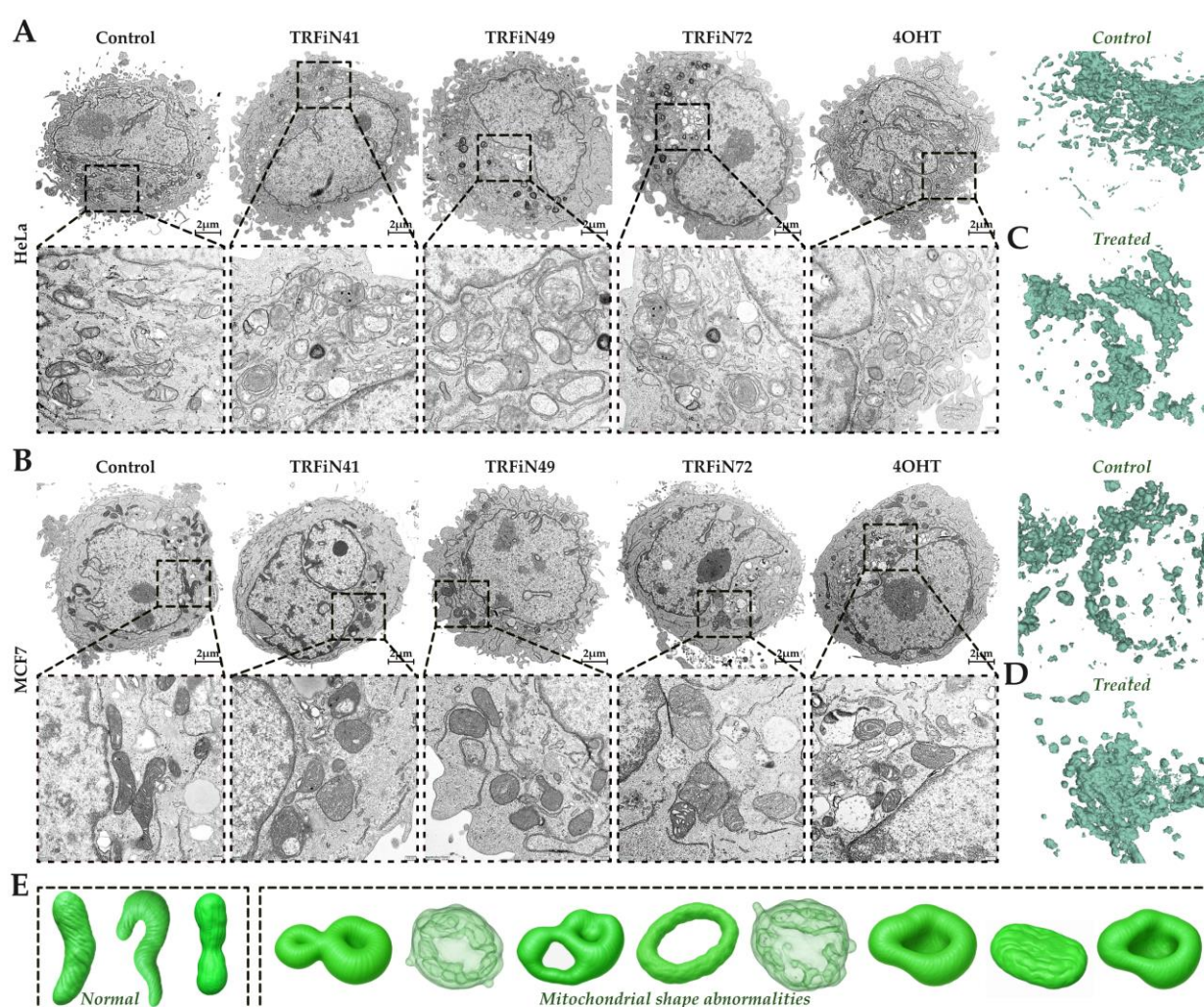


Figure 5.12. *Effect of TRFiN compounds and 4OHT on mitochondrial ultrastructure in HeLa and MCF7 cells.* (A,B) Representative transmission electron microscopy images of HeLa and MCF7 cells, respectively, after treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT, with untreated cells as control. (C,D) Airyscan 3D processed views of representative control and treated mitochondria in HeLa and MCF7 cells, respectively. (E) Reconstructed mitochondrial images showing mitochondrial morphology based on the transmission electron microscopy images.

The same general changes were present in MCF7 cells. Control mitochondria showed a more regular shape and preserved internal structure, whereas the treated groups contained swollen and

abnormal mitochondrial profiles with less regular cristae and altered internal appearance (Figure 5.12B). These ultrastructural changes were therefore present in both cell lines and were consistent across the different treatments. The Airyscan 3D processed views showed the same structural shift. In both HeLa and MCF7 cells, representative control mitochondria had a more extended and connected appearance, while treated mitochondria appeared more compact, fragmented, and irregular (Figure 5.12C,D). The reconstructed mitochondrial forms based on TEM morphology showed this change more clearly. Control reconstructions showed the expected elongated and curved mitochondrial forms, whereas treated reconstructions showed several abnormal forms, including rounded, ring like, donut like, swollen, and hollow structures (Figure 5.12E). These reconstructed shapes matched the abnormalities seen in the TEM images and showed the range of mitochondrial forms present after treatment. The functional and structural analyses therefore showed the same pattern in both cell lines. The JC-1 assay showed time dependent loss of mitochondrial membrane potential. MitoTracker Green imaging showed fragmentation and collapse of the normal mitochondrial network. Transmission electron microscopy showed swelling, rounding, disturbed cristae, and loss of the regular elongated mitochondrial profile. The reconstructed forms based on TEM morphology showed the same shift from normal elongated mitochondria to abnormal rounded and ring like structures. The timing of this response differed between the two cell lines. HeLa cells showed an earlier functional change, with a reduced JC-1 ratio already visible at 24 h in TRFiN41- and TRFiN72 treated cells, while MCF7 cells showed little change at 24 h and a clearer decrease from 72 h onward. At later time points, TRFiN41 produced the strongest drop in the JC-1 ratio in HeLa cells and one of the strongest drops in MCF7 cells. 4OHT also caused a strong reduction in both models. TRFiN49 showed a moderate effect that became stronger with time, while TRFiN72 showed a marked decrease at 120 h but a weaker late reduction than TRFiN41 and 4OHT. These results show that the tested compounds caused time dependent mitochondrial injury in both HeLa and MCF7 cells.

5.10 Induction of apoptosis and caspase-3/7 activation after treatment with TRFiN compounds

Apoptotic cell death was examined after 24, 72, 120, and 168 h of treatment using annexin V and 7-AAD staining. The analysis separated the cell populations into live, early apoptotic, late apoptotic, and debris or dead fractions. Representative annexin V/7-AAD dot plots for HeLa and MCF7 cells and the corresponding quantified populations are presented in Figure 5.13A-D. Caspase-3/7 activity was examined separately in HeLa cells using CellEvent™ Caspase-3/7 Green Detection Reagent, while this analysis was not performed in MCF7 cells because these cells lack functional caspase-3. In HeLa cells, the annexin V/7-AAD distribution remained close to the untreated control during the first 24 h. More than 94% of cells remained in the live fraction in both control and treated groups, while the early and late apoptotic populations remained low. The similar distribution of cells across the four quadrants in Figure 5.13A showed that none of the treatments caused a clear early apoptotic shift in HeLa cells. A change became visible after 72 h, when the live fraction decreased more clearly under TRFiN41 and 4OHT than under TRFiN49 and TRFiN72. This decrease was accompanied mainly by an increase in late apoptosis, which rose from 3.9% in the control to 8.3% under TRFiN41 and 7.0% under 4OHT. TRFiN49 and TRFiN72 produced smaller late apoptotic fractions of approximately 5-6%. The early apoptotic population remained low across the treatments, showing that the main change in HeLa cells at this time point occurred in the late apoptotic compartment, as shown in the quantified distributions in Figure 5.13B.

The apoptotic response in HeLa cells became more evident after 120 and 168 h of treatment. At 120 h, the live population remained close to 89% in the untreated control but decreased to approximately 75-82% in the treated groups. The greatest loss of live cells was observed after 4OHT treatment, followed by TRFiN41 and TRFiN49, while TRFiN72 produced a smaller decrease. At the same time, the late apoptotic fraction increased to approximately 10-14%, corresponding to an increase of about 1.8- to 2.5-fold over the control. TRFiN41 produced the highest late apoptotic population at this stage, whereas TRFiN49 showed an intermediate response. The early apoptotic fraction remained

low under the three TRFiN compounds but increased more clearly under 4OHT. By 168 h, the movement of treated HeLa cells out of the live quadrant was marked in the dot plots presented in Figure 5.13A. Only approximately 54-60% of treated cells remained live compared with 84.5% in the control. The late apoptotic population increased to approximately 28-32% under all treatments, representing an almost sixfold increase over the control. TRFiN41 produced the highest late apoptotic fraction, followed closely by 4OHT, TRFiN72, and TRFiN49. The early apoptotic population remained below 11% and did not increase uniformly across the treatment groups. The debris or dead fraction also increased during prolonged exposure, although it remained smaller than the late apoptotic population. The quantified values in Figure 5.13B therefore show that the principal late response in HeLa cells was a large reduction in the live population together with an increase in late apoptosis.

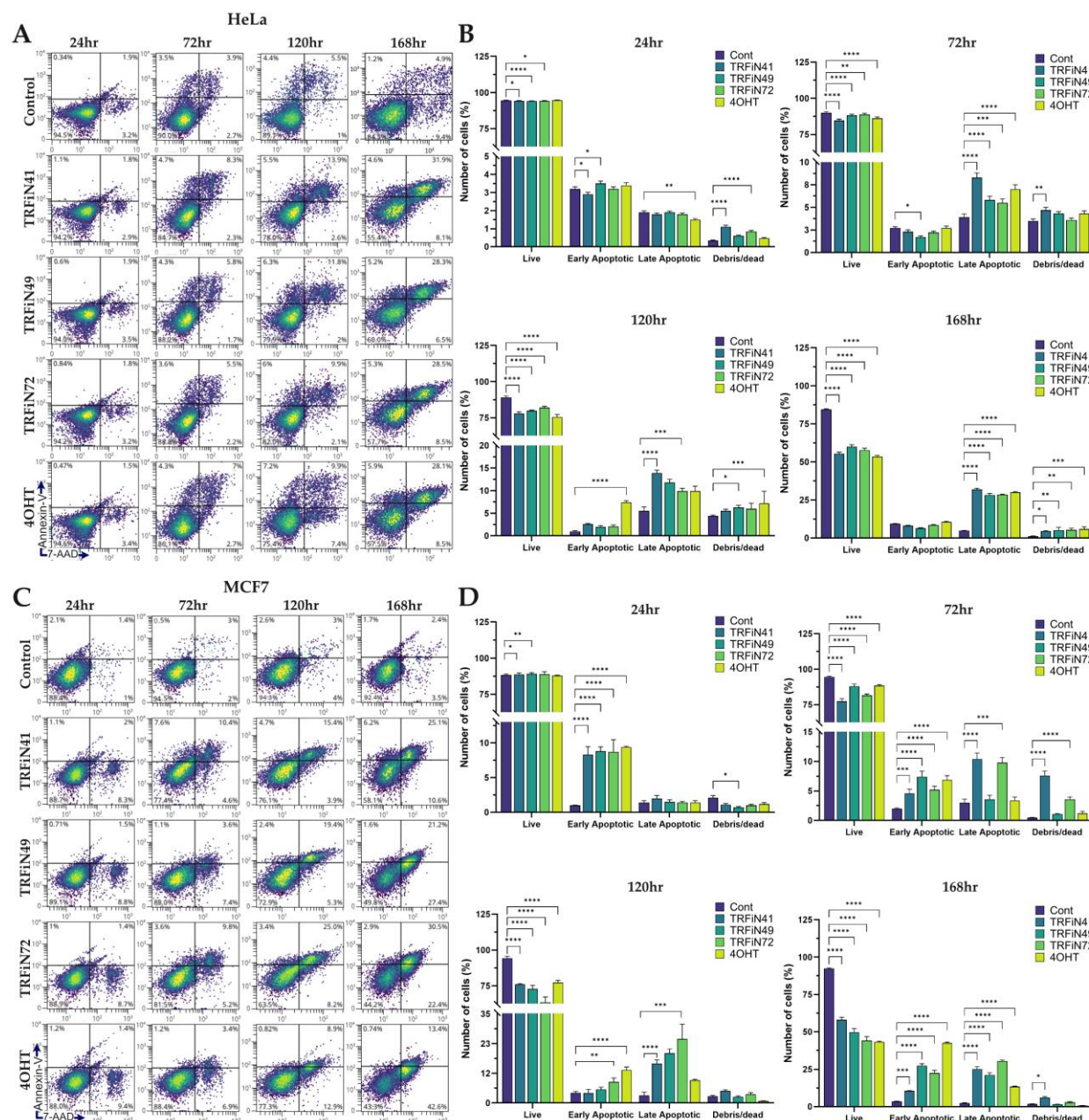


Figure 5.13. Effect of TRFiN compounds and 4OHT on apoptosis in HeLa and MCF7 cells. (A,C) Representative annexin V/7-AAD dot plots of HeLa and MCF7 cells, respectively, at different time points after treatment, with untreated cells as control. (B,D) Quantification of live, early apoptotic, late apoptotic, and debris or dead cell populations in HeLa and MCF7 cells, respectively. Data represent mean $\pm$ standard deviation ($n = 3$). Statistical analysis was conducted by two way analysis of variance and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

MCF7 cells showed a different distribution during the early treatment period. At 24 h, the live fraction remained close to 88-89% in all groups, and the late apoptotic population remained below 2%. However, the early apoptotic fraction increased from approximately 1% in the control to between 8.3% and 9.4% following treatment. Similar increases were produced by all four treatments, with 4OHT giving the highest value, followed closely by TRFiN49, TRFiN72, and TRFiN41. The representative dot plots in Figure 5.13C show this movement towards the annexin V positive and 7-AAD negative quadrant without a corresponding loss of the main live population. At 72 h, the treatment responses separated more clearly. TRFiN41 and TRFiN72 reduced the live fraction to approximately 77% and 82%, respectively, and increased late apoptosis to approximately 10%. These late apoptotic values were more than threefold higher than the control. In comparison, TRFiN49 and 4OHT retained live populations close to 88%, while their late apoptotic fractions remained near the control. The early apoptotic population remained elevated, particularly under TRFiN49 and 4OHT. The quantified results in Figure 5.13D therefore show that TRFiN41 and TRFiN72 produced a greater movement into late apoptosis at 72 h, whereas TRFiN49 and 4OHT retained a larger proportion of cells in the early apoptotic compartment.

Longer treatment caused a pronounced reduction in the live MCF7 population. At 120 h, approximately 94% of control cells remained live, whereas the treated groups contained approximately 64-77% live cells. TRFiN72 produced the largest decrease and the highest late apoptotic population, which reached 25%. TRFiN49 and TRFiN41 produced intermediate late apoptotic fractions, while 4OHT produced the lowest late apoptotic fraction but the highest early apoptotic population. These differences became greater at 168 h, when only approximately 43-58% of treated MCF7 cells remained live compared with more than 92% of control cells. TRFiN72 produced the highest late apoptotic fraction at approximately 31%, followed by TRFiN41 and TRFiN49. In contrast, 4OHT produced a smaller late apoptotic population but a marked accumulation of early apoptotic cells, which reached approximately 43%. TRFiN49 and TRFiN72 also produced large early apoptotic populations, while the increase under TRFiN41 was smaller. The final MCF7 distributions in Figure 5.13C,D show that the three TRFiN compounds and 4OHT did not produce identical movement between the early- and late apoptotic compartments. TRFiN72 was associated mainly with a high late apoptotic fraction, whereas the 4OHT treated population remained concentrated more strongly in early apoptosis.

Caspase-3/7 activation in HeLa cells also changed with treatment duration. The representative histograms and quantified caspase positive populations are presented in Figure 5.14A,B. At 24 h, TRFiN41 and TRFiN49 produced moderate increases in the caspase positive population, while the responses to TRFiN72 and 4OHT remained smaller. At 72 h, TRFiN41 produced the clearest increase, reaching approximately 18% caspase positive cells compared with approximately 10% in the control. TRFiN49 produced a smaller increase, whereas TRFiN72 remained close to the control and 4OHT was below the corresponding control value. Caspase activity increased further at 120 h, when TRFiN41 and 4OHT produced caspase positive fractions of approximately 23-25%. TRFiN49 showed an intermediate value, while TRFiN72 again produced a smaller increase. The strongest caspase-3/7 response was recorded after 168 h. At this time point, approximately 33-46% of treated cells were caspase positive compared with 11% in the control. TRFiN41 produced the highest response, followed by TRFiN49, TRFiN72, and 4OHT. The marked rightward shifts in the 168 h histograms in Figure 5.14A corresponded with the large increase in the quantified caspase positive fractions shown in Figure 5.14B.

Changes in mitochondrial organisation were examined by immunofluorescence staining of translocase of outer mitochondrial membrane 20 (TOMM20) and AIF. In untreated MCF7 cells, TOMM20 and AIF formed a mainly continuous mitochondrial pattern around the nucleus, with considerable overlap between the two signals. Following treatment, the mitochondrial staining became more fragmented and unevenly distributed, as shown in Figure 5.14C. TRFiN41, TRFiN49, TRFiN72, and 4OHT produced areas of clustered perinuclear staining together with regions showing

reduced continuity of the TOMM20 labelled network. The enlarged images also showed changes in the spatial overlap of TOMM20 and AIF, with partial separation of the green and red signals in several treated cells. In untreated HeLa cells, TOMM20 and AIF also showed a continuous and overlapping mitochondrial distribution. Treatment altered this organisation and produced stronger accumulation of mitochondrial staining around the nucleus, together with less uniform TOMM20 and AIF signals in Figure 5.14D. These changes were particularly clear following TRFiN41, TRFiN49, and 4OHT treatment. TRFiN72 also altered mitochondrial organisation, although the staining pattern was more variable between cells and retained areas of both overlapping and separated TOMM20 and AIF signals.

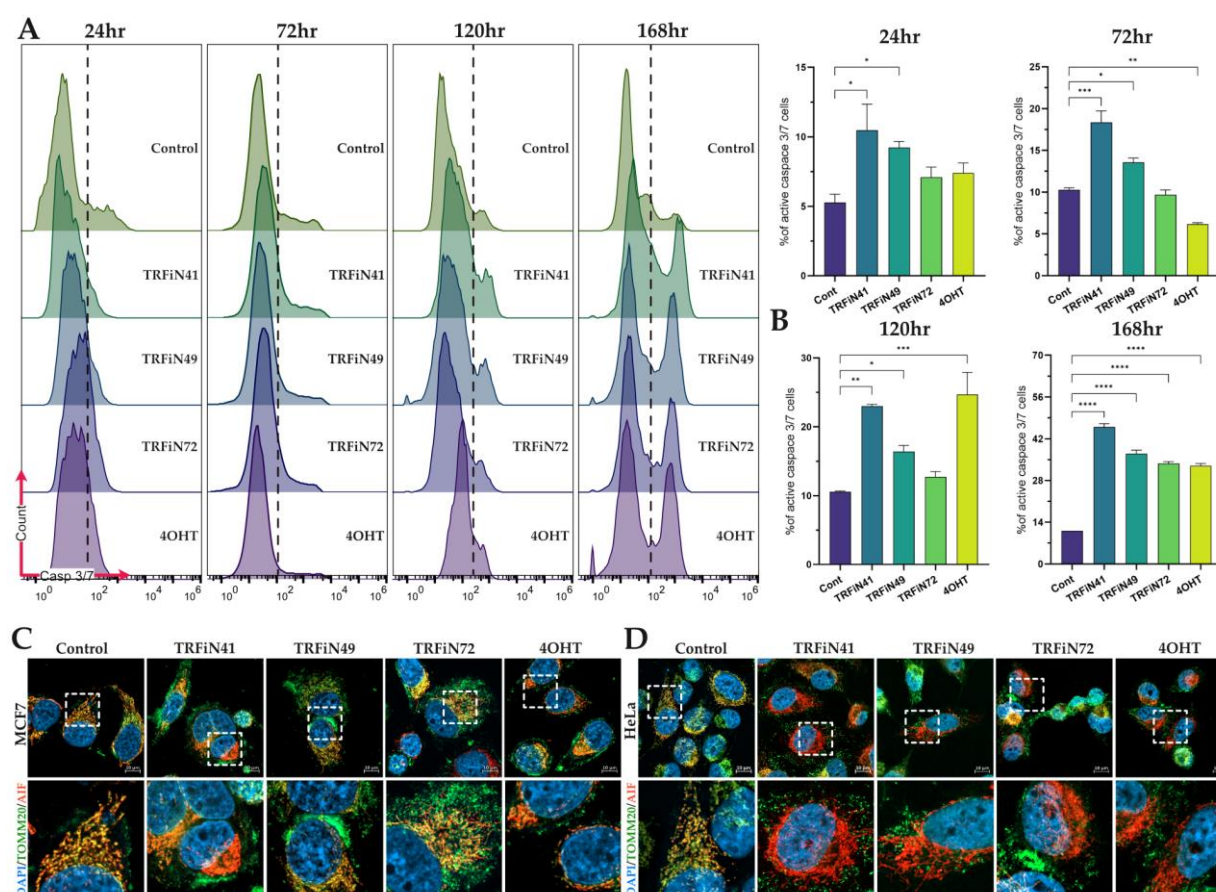


Figure 5.14. Effect of TRFiN compounds and 4OHT on caspase-3/7 activation and mitochondrial apoptosis associated changes in HeLa and MCF7 cells. (A) Representative flow cytometric histograms of active caspase-3/7 in HeLa cells at different time points after treatment, with untreated cells as control. (B) Quantification of caspase-3/7 positive HeLa cells after treatment. (C,D) Representative immunofluorescence images showing TOMM20 and apoptosis inducing factor (AIF) staining in MCF7 and HeLa cells, respectively, after treatment. Data represent mean $\pm$ standard deviation ($n = 3$). Statistical analysis was conducted by two way analysis of variance and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

5.11 Treatment with TRFiN compounds promotes progressive senescence in MCF7 and HeLa cells

Cellular senescence was measured after 24, 72, 120, and 168 h of treatment using the CellEvent Senescence Green Flow Cytometry Assay. Representative fluorescence histograms for MCF7 and HeLa cells are presented in Figure 5.15A and B, respectively, while the quantified senescent populations are shown in Figure 5.15E and F. The flow cytometry measurements were supported by X-gal staining at 168 h, together with Hoechst 33342 staining to show the nuclei in the same microscopic fields. The untreated populations remained low throughout the experiment in both cell lines, with senescent fractions generally remaining between approximately 5% and 6%. The treated populations were also close to this range at 24 h. No clear separation between the control and treated histograms was observed at this early time point in either cell line. A marked rightward shift in senescence associated fluorescence became visible from 72 h onward and increased further at 120 and

168 h. In MCF7 cells, all four treatments produced a clear increase in the senescent population after 72 h, as shown in Figure 5.15A,E. The treated fractions ranged from approximately 29% to 42%, compared with about 5% in the control. TRFiN41 produced the highest response at this time point, followed by TRFiN49, 4OHT, and TRFiN72. This represented an approximately 5.5-to 8.1-fold increase over the control. The separation between the treated and untreated populations became greater at 120 h, when nearly half to approximately two thirds of the treated cells were senescence positive. TRFiN41 again produced the highest fraction at about 65%, while TRFiN49 and 4OHT produced similar intermediate values and TRFiN72 produced the lowest treated value. Despite this lower response, the senescent population under TRFiN72 remained more than sevenfold above the control. At 168 h, senescence increased further across all treatment groups. TRFiN41 reached approximately 79%, TRFiN49 and 4OHT reached about 70% and 68%, respectively, and TRFiN72 reached 62%, while the control remained at approximately 6%. The MCF7 histograms in Figure 5.15A show the gradual separation of the treatment distributions from the control over time, with TRFiN41 producing the strongest shift from 72 h onward.

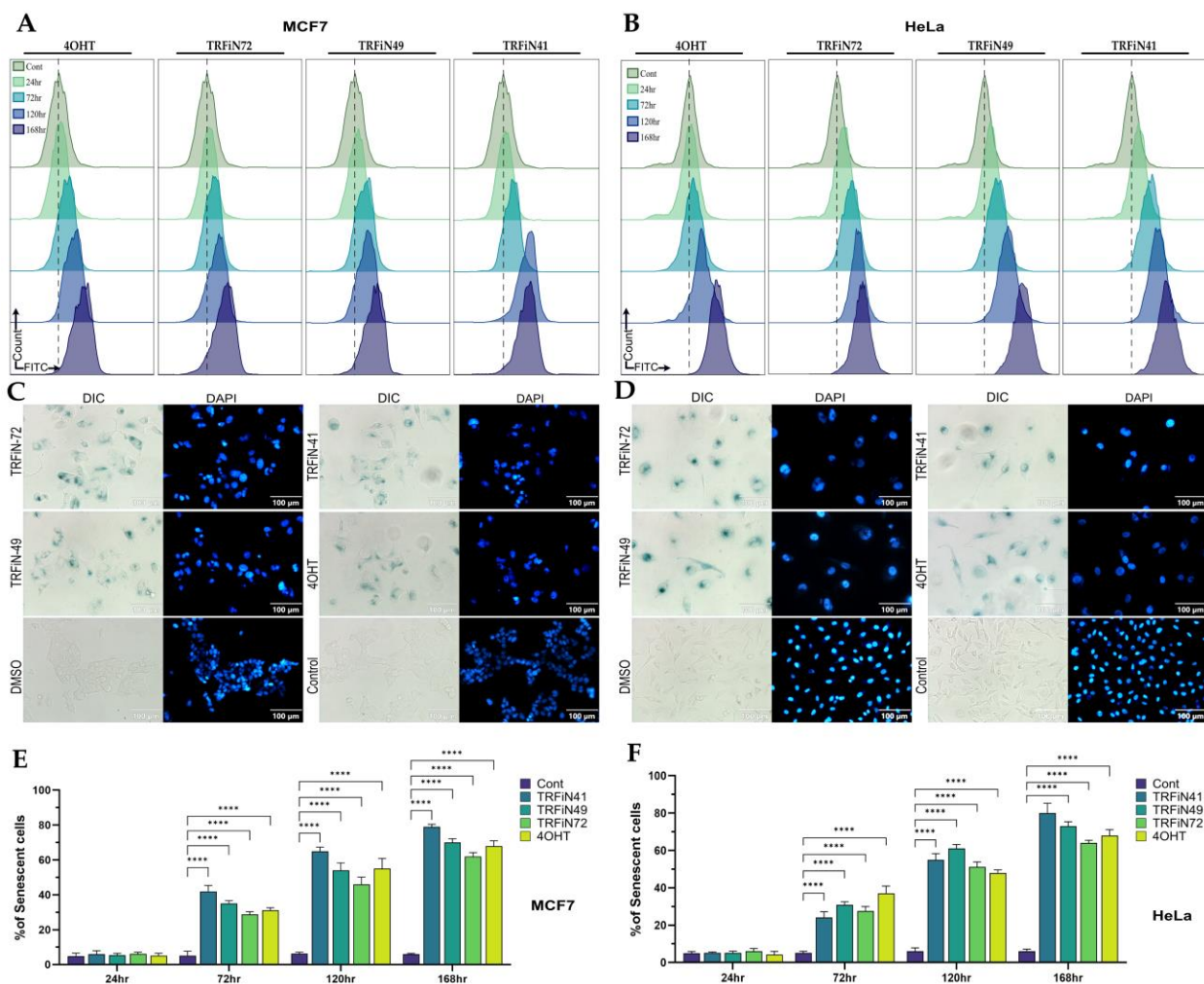


Figure 5.15. Effect of TRFiN compounds and 4OHT on senescence in MCF7 and HeLa cells. (A,B) Representative flow cytometric histograms of senescence associated fluorescence in MCF7 and HeLa cells, respectively, at 24, 72, 120, and 168 h after treatment, with untreated cells as control. (C,D) Representative X-gal staining and DAPI nuclear staining of MCF7 and HeLa cells, respectively, at 168 h. (E,F) Quantification of senescent cells in MCF7 and HeLa cells, respectively, at different time points after treatment. Data represent mean $\pm$ SD ($n = 3$). Statistical analysis was conducted by two way analysis of variance and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

HeLa cells showed a similarly delayed increase, although the relative order of the treatments changed between the time points. The control and treated populations remained close to 5% at 24 h, with no clear early increase in senescence associated fluorescence. At 72 h, the senescent fractions increased to approximately 24-37%, corresponding to about 4.7-to 7.3-fold higher values than the

control. Unlike MCF7 cells, 4OHT produced the highest response in HeLa cells at this time point, followed by TRFiN49 and TRFiN72, while TRFiN41 produced the lowest treated value. A further increase was observed at 120 h in Figure 5.15B,F, when approximately 48-61% of treated cells were classified as senescent. TRFiN49 produced the highest value at this stage, followed by TRFiN41 and TRFiN72, while 4OHT produced a slightly smaller response. By 168 h, TRFiN41 had become the strongest treatment and increased the senescent fraction to approximately 80%. TRFiN49 reached about 73%, followed by 4OHT at 68% and TRFiN72 at 64%. These final values were approximately 10.5- to 13.2-fold higher than the corresponding control. The progressive rightward movement of the HeLa treatment histograms in Figure 5.15B followed the increase shown in the quantified data in Figure 5.15F. The microscopic staining at 168 h showed clear differences between the control and treated populations. The control and dimethyl sulfoxide groups displayed little X-gal staining and retained the usual cell shape in both cell lines. In contrast, the treated groups contained numerous X-gal positive cells with enlarged and flattened morphology, as shown for MCF7 and HeLa cells in Figure 5.15C and D. In MCF7 cells, the blue green staining and spreading of the cells were particularly clear under TRFiN41 and TRFiN49. TRFiN72 and 4OHT also produced visible X-gal positive populations, although the staining appeared less extensive than under TRFiN41. Treated HeLa cells also showed stronger X-gal staining than the control groups, together with increased cell size and a broader, flattened appearance. The Hoechst 33342 images showed intact nuclei within the stained cells and allowed the X-gal positive cell bodies to be distinguished from cell debris in the corresponding fields.

5.12 TRFiN compounds suppress cell migration in a dose dependent manner in MCF7 and HeLa cells

Cell migration was examined using a wound healing assay in which the movement of the opposing cell fronts was followed for 72 h during continuous treatment with TRFiN41, TRFiN49, and TRFiN72. The compounds were examined at concentrations of 2, 5, and 10 μM , while sorafenib was included as a reference inhibitor at 4.5 μM . Cell front velocity was used to compare the rate of movement between the control and treated populations, and the percentage of wound closure was measured throughout the observation period. The representative phase contrast images in Figure 5.16A and F show the MCF7 and HeLa wounds, respectively, after treatment with the TRFiN compounds at 2 μM and sorafenib at 4.5 μM . Under the control condition, the cell fronts moved steadily towards the centre of the wound, resulting in almost complete closure by the end of the observation period in both cell lines. Treatment delayed this movement, although the degree of inhibition depended on the compound concentration and the cell line. MCF7 cells showed a clear concentration dependent reduction in cell front velocity. The control populations moved at approximately 3.7 $\mu\text{m}/\text{h}$ in each compound series, while treatment at 2 μM produced only small changes. A clearer decrease was recorded at 5 μM , where all three TRFiN compounds reduced migration velocity by approximately one quarter to one third relative to their corresponding controls. TRFiN49 produced the largest reduction at this intermediate concentration, followed by TRFiN41 and TRFiN72. The effect became stronger at 10 μM , as shown by the velocity measurements in Figure 5.16B. At this concentration, TRFiN41 reduced cell front velocity by approximately 52%, TRFiN49 by approximately 42%, and TRFiN72 by approximately 33%. Sorafenib produced the lowest velocity and reduced migration by approximately 64% relative to the control. The distribution of the velocity measurements therefore showed a progressive decrease with increasing concentration, with TRFiN41 producing the greatest reduction among the TRFiN compounds at the highest tested concentration. The representative MCF7 images in Figure 5.16A also showed a treatment related delay at the lower concentration. Although the control wound was almost closed by 72 h, a residual gap remained in the treated populations. The delay was more visible under TRFiN41 and TRFiN49 than under TRFiN72, while sorafenib retained a broad open area at the final time point.

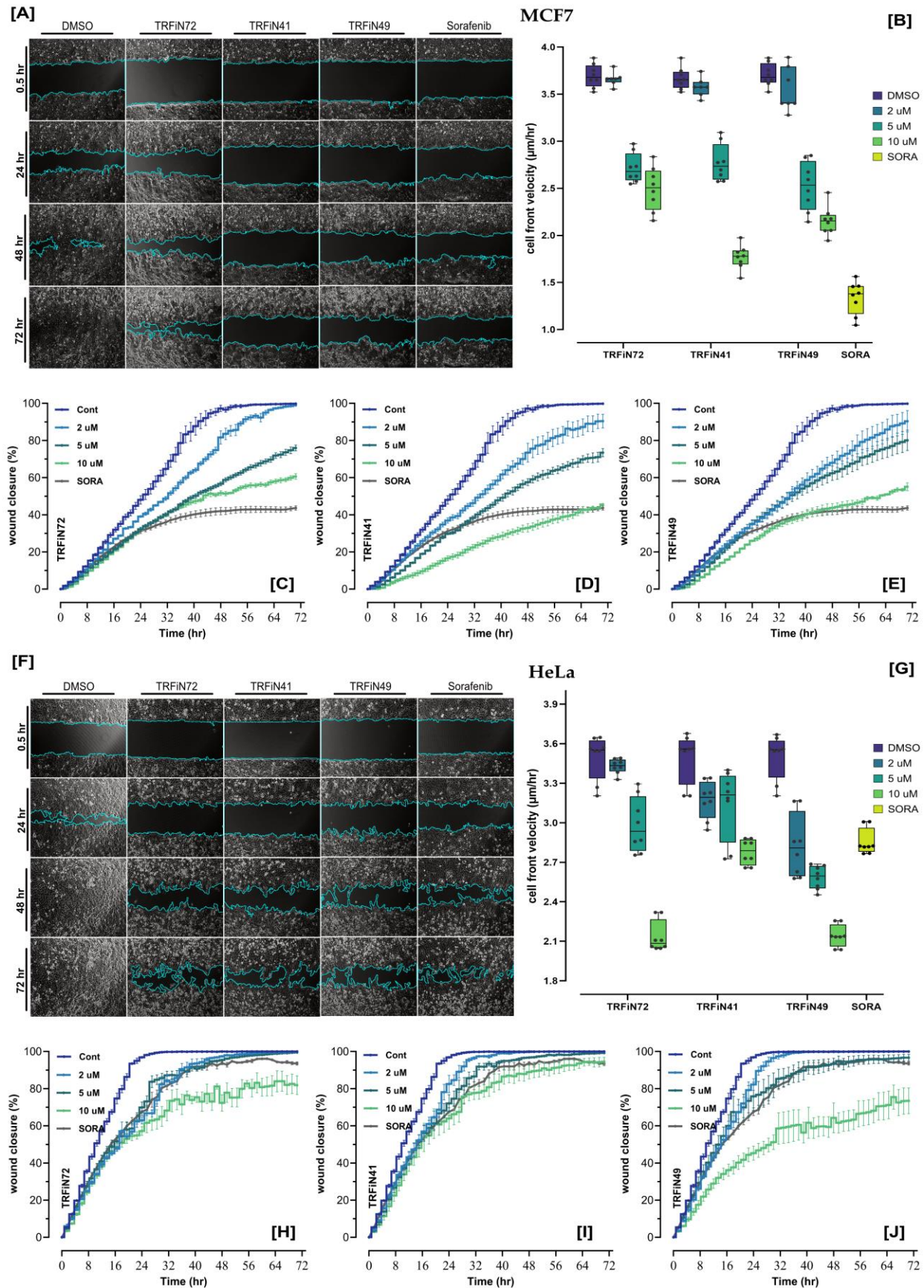


Figure 5.16. Effect of TRFiN compounds and sorafenib on wound healing and migration in MCF7 and HeLa cells. (A,F) Representative wound closure images of MCF7 and HeLa cells, respectively. (B,G) Cell front velocity in MCF7 and HeLa cells, respectively. (C E) Concentration dependent wound closure of TRFiN72, TRFiN41, and TRFiN49 in MCF7 cells. (H J) in HeLa cells. Data represent mean $\pm$ SD. Statistical analysis was conducted by two way analysis of variance and post hoc Dunnett's test.

The complete concentration response curves in Figure 5.16C-E showed that the separation from the control increased as the concentration increased. At 2 μM , TRFiN72 remained close to the control, whereas TRFiN41 and TRFiN49 caused a more visible delay. At 5 μM , all three compounds reduced closure, but the wound still closed by more than 70% during the 72 h period. The greatest separation was recorded at 10 μM . Under this condition, the final wound closure was approximately 60% with TRFiN72, 55% with TRFiN49, and 45% with TRFiN41, compared with almost complete closure in the control. Sorafenib retained wound closure at approximately 43-45% and remained the strongest inhibitor in the MCF7 assay. HeLa cells showed smaller changes than MCF7 cells at the low and intermediate concentrations. The control cell fronts moved at approximately 3.5 $\mu\text{m}/\text{h}$ across the three compound series. At 2 μM , TRFiN72 produced little change in velocity, while TRFiN41 caused a moderate decrease. TRFiN49 showed the clearest effect at this concentration and reduced the movement of the HeLa cell front by approximately 19%. A similar pattern was recorded at 5 μM . TRFiN72 and TRFiN41 produced only limited additional reductions, whereas the effect of TRFiN49 remained more apparent. The velocity measurements in Figure 5.16G therefore showed that TRFiN49 affected HeLa migration at a lower concentration than the other two TRFiN compounds.

The differences between the HeLa treatment groups became clearer at 10 μM . TRFiN72 and TRFiN49 each reduced cell front velocity by approximately 39% relative to their controls, while TRFiN41 produced a smaller reduction of approximately 21%. Sorafenib reduced HeLa cell front velocity only moderately and remained less effective than TRFiN72 and TRFiN49 at 10 μM . In the representative images obtained at the lower TRFiN concentration, the treated HeLa populations retained visible residual wound areas at 72 h, whereas the control wound was almost completely closed in Figure 5.16F. The delay was most visible under TRFiN49 and sorafenib, while TRFiN72 and TRFiN41 produced smaller changes at this concentration. The HeLa wound closure curves in Figure 5.16H-J showed that TRFiN72 and TRFiN41 caused a clear delay mainly at the highest concentration. At 2 and 5 μM , their curves remained relatively close to the control and approached almost complete closure by 72 h. At 10 μM , TRFiN72 limited final closure to approximately 83%, whereas TRFiN41 produced a smaller delay and allowed approximately 94% closure. TRFiN49 showed a clearer concentration dependent response, with separation from the control already visible at 2 and 5 μM . At 10 μM , TRFiN49 produced the greatest delay among the three compounds and limited wound closure to approximately 74%. Sorafenib remained close to the control range in the HeLa closure curves and produced only a modest reduction compared with its stronger effect in MCF7 cells.

5.13 TRFiN compounds suppress spheroid growth in three dimensional culture and alter spheroid viability

Three dimensional spheroid growth was examined to assess the response of MCF7 and HeLa cells under conditions that better preserve cell cell contact and spatial organisation than monolayer culture. The spheroids were followed from 3 h to 120 h after treatment with TRFiN41, TRFiN49, TRFiN72, and 4OHT. In both cell lines, the untreated spheroids increased progressively in size during the observation period, whereas treated spheroids showed reduced expansion. The extent of inhibition depended on the compound, the concentration, and the cell line.

In MCF7 spheroids, the control continued to enlarge across the full time course, and the final increase in spheroid area was about 0.016 to 0.018 mm^2 above the starting value, depending on the individual series. TRFiN41 produced a clear concentration dependent reduction in growth. At 5 μM , the final increase in spheroid area remained relatively close to the control, reaching about 0.012 mm^2 by the end of the experiment. At 10 μM , the increase was much smaller, at about 0.002 mm^2 . At 20 μM , the spheroid area remained near the starting level and showed a slight decline relative to baseline, while 30 μM also kept the spheroid close to its starting size. The 4OHT control produced the strongest

suppression in this series and remained almost flat throughout the experiment, with only a minimal change in spheroid area. The bright field images show the same pattern. In the control wells, the MCF7 spheroids became progressively larger and denser with time, whereas higher concentrations of TRFiN41 and 4OHT clearly limited the increase in spheroid size, particularly at 72 h and 120 h (Figure 5.17A). TRFiN49 also suppressed MCF7 spheroid growth, but the response was slightly different. At 5 μM , the spheroid growth curve remained close to the control and reached a final increase of about 0.018 mm^2 . At 10 μM , the increase was lower, at about 0.013 mm^2 . At 20 μM and 30 μM , the final increase remained much smaller, around 0.002 to 0.004 mm^2 . The 4OHT curve again stayed almost flat. This shows that TRFiN49 also reduced spheroid growth in a concentration dependent manner, but the inhibitory effect at 5 μM was weaker than for TRFiN41. The image series supports this interpretation. The MCF7 spheroids treated with 20 μM and 30 μM TRFiN49 remained smaller and more compact than the control by 120 h, whereas the 5 μM spheroids retained a growth pattern closer to untreated cells (Figure 5.17A).

TRFiN72 behaved differently in MCF7 spheroids. At 5 μM , spheroid growth was not suppressed and instead exceeded the control by the end of the experiment, reaching a final increase of about 0.024 mm^2 . At 10 μM , the final increase was about 0.013 mm^2 , which was below the control but still much higher than the 10 μM values in the TRFiN41 series. At 20 μM and 30 μM , the growth curves were irregular. Both concentrations showed partial suppression compared with the control, but neither produced the strong inhibition seen with TRFiN41 or TRFiN49 at the same doses. The final increases remained around 0.008 to 0.010 mm^2 . The 4OHT control again remained low and nearly flat. The MCF7 data therefore show that TRFiN72 was less effective than TRFiN41 and TRFiN49 in this model and that its effect was less regular across concentrations. This weaker response is also visible in the image series, where the TRFiN72 treated spheroids retained substantial growth over time and did not contract to the same extent as those treated with higher doses of TRFiN41 or exposed to 4OHT (Figure 5.17A). The HeLa spheroids showed a stronger overall response to treatment than the MCF7 spheroids. In the control condition, the final increase in spheroid area reached about 0.130 mm^2 by 120 h in all three series. TRFiN41 reduced this increase in a clear concentration dependent manner. At 5 μM , the final increase remained high, at about 0.121 mm^2 , but at 10 μM it fell to about 0.090 mm^2 , at 20 μM to about 0.076 mm^2 , and at 30 μM to about 0.033 mm^2 . The 4OHT group showed the strongest suppression and remained almost flat, with only a small final increase of about 0.015 mm^2 . The HeLa images reflect this pattern. The control spheroids became progressively larger from 3 h to 120 h, whereas the higher TRFiN41 concentrations, especially 20 μM and 30 μM , clearly restricted spheroid growth, and 4OHT kept the spheroids small throughout the experiment (Figure 5.17B). TRFiN49 produced the strongest effect in HeLa spheroids. At 5 μM , the final increase in spheroid area was about 0.085 mm^2 , already well below the control. At 10 μM , the final increase fell further to about 0.045 mm^2 . At 20 μM and 30 μM , the curves remained very low, close to 0.016 to 0.020 mm^2 by the end of the experiment. The 4OHT response again remained almost flat. This pattern indicates that HeLa spheroids were particularly sensitive to TRFiN49, and that even the lowest dose produced substantial growth suppression in this three dimensional system. The image series supports this, as the TRFiN49 treated HeLa spheroids remained visibly smaller than the control across the whole time course and showed little enlargement at 20 μM and 30 μM (Figure 5.17B).

TRFiN72 also reduced HeLa spheroid growth in a concentration dependent manner, although the effect was weaker than TRFiN49. At 5 μM , the final increase remained about 0.100 mm^2 , while 10 μM reduced it to about 0.056 mm^2 . At 20 μM and 30 μM , the final increase was about 0.033 mm^2 and 0.032 mm^2 , respectively. The 4OHT curve again remained almost flat at about 0.015 mm^2 . Thus, TRFiN72 clearly suppressed HeLa spheroid growth, especially from 10 μM onward, but the degree of inhibition remained less than with TRFiN49. When the HeLa curves are compared directly, the ranking of growth suppression was most pronounced for TRFiN49, followed by TRFiN72 and then TRFiN41, while 4OHT remained the strongest inhibitory reference (Figure 5.17B). The confocal images acquired after 120 h provide additional information on spheroid structure and cell state. In both MCF7

and HeLa control spheroids, the blue Hoechst 33342 signal marked a dense and compact spheroid body, and the overall architecture remained relatively uniform. In the treated spheroids, the organisation was less compact and the distribution of annexin V and 7-aminoactinomycin D (7-AAD) signals was broader. In MCF7 spheroids, TRFiN41 and TRFiN49 were associated with loss of the compact spheroid outline and more widespread green annexin V and red 7-AAD staining than in the control. TRFiN72 also changed spheroid structure, but the degree of disruption appeared lower than with TRFiN41 and TRFiN49. The 4OHT spheroids showed strong loss of compact organisation with prominent non-blue signal. A similar pattern was present in HeLa spheroids. The control remained dense and rounded, whereas treated spheroids showed a looser structure and wider distribution of annexin V positive and 7-AAD positive cells. In HeLa, this was especially marked under TRFiN49 and 4OHT, which is in line with the strong reduction in spheroid growth seen in the time course results (Figure 5.17C,D).

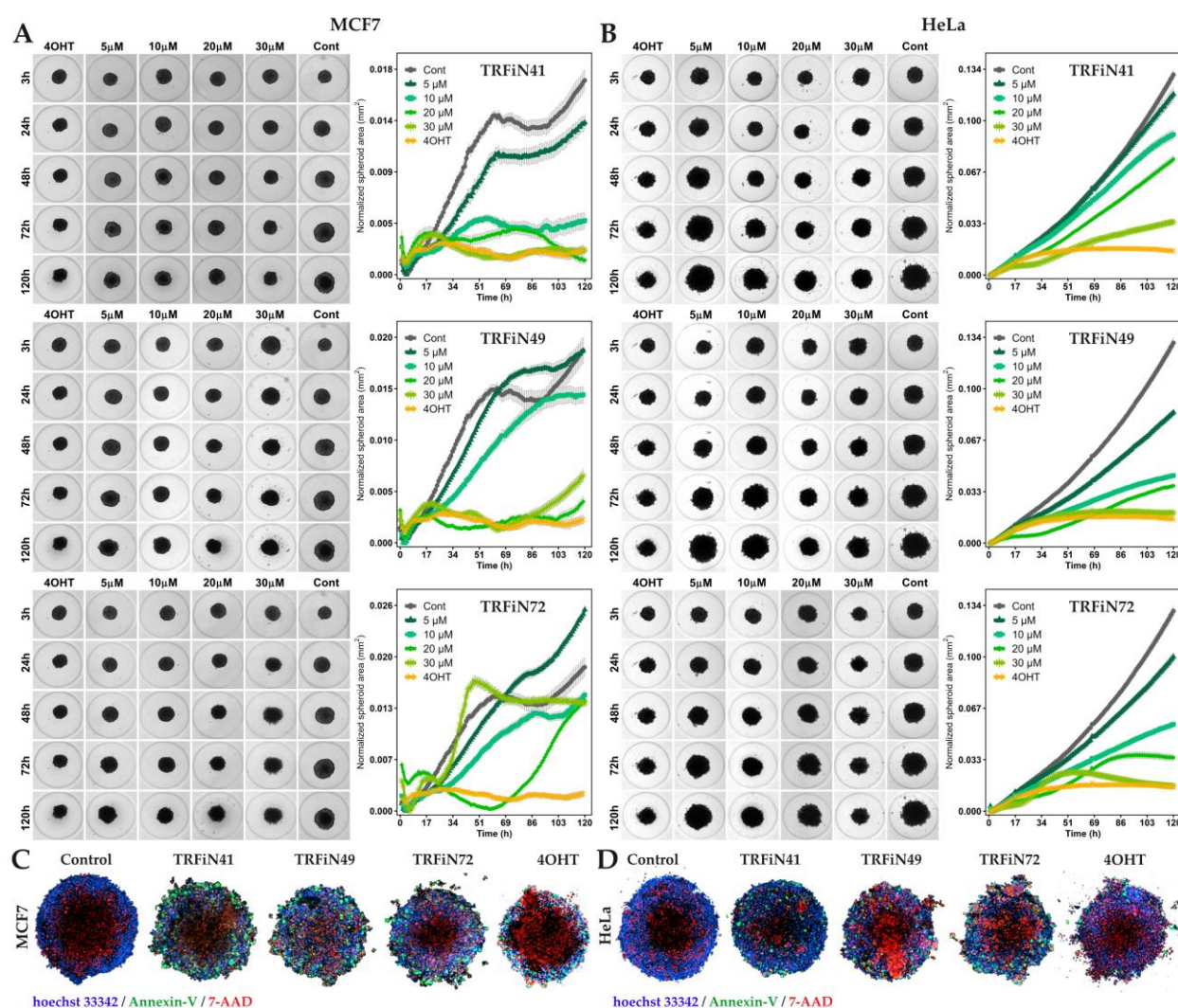


Figure 5.17. Effect of TRFiN compounds and 4-hydroxytamoxifen on spheroid growth and spheroid viability in MCF7 and HeLa cells. (A,B) Time dependent spheroid growth of MCF7 and HeLa cells, respectively, after treatment with TRFiN41, TRFiN49, TRFiN72, and 4-hydroxytamoxifen at different concentrations. (C,D) Representative three dimensional confocal images of MCF7 and HeLa spheroids, respectively, after 120 h of treatment, stained with Hoechst 33342, annexin V, and 7-aminoactinomycin D.

5.14 TRFiN compounds reduce clonogenic growth and show cell line dependent accumulation of acidic vesicular organelles

The long term proliferative capacity of HeLa and MCF7 cells was examined using a colony formation assay after treatment with increasing concentrations of TRFiN41, TRFiN49, TRFiN72, and 4OHT. Colony area was used to determine whether the cells retained the ability to survive treatment

and form new colonies over an extended period. The representative crystal violet stained plates and quantified colony areas are presented in Figure 5.18A and B. A concentration dependent reduction in colony formation was observed in both cell lines, although the strength of the response differed between the compounds. In HeLa cells, TRFiN49 produced a marked reduction from the lowest tested concentrations and became the strongest inhibitor among the three TRFiN compounds. At 12 μM , TRFiN49 had already reduced colony area by more than 99%, and only a very small residual area remained at 20 μM . Treatment with 4OHT produced a similarly strong response at the higher concentrations, reducing colony formation by approximately 95% at 12 μM and by more than 98% at 20 μM . TRFiN41 caused a more gradual decline across the concentration range. Its effect was moderate at the lower concentrations but became pronounced at 12 μM , and colony formation was almost completely lost at 20 μM . TRFiN72 showed the weakest response in HeLa cells. Although the colony area decreased as the concentration increased, visible colonies remained even at the highest concentration, where the reduction was approximately 63% relative to its control.

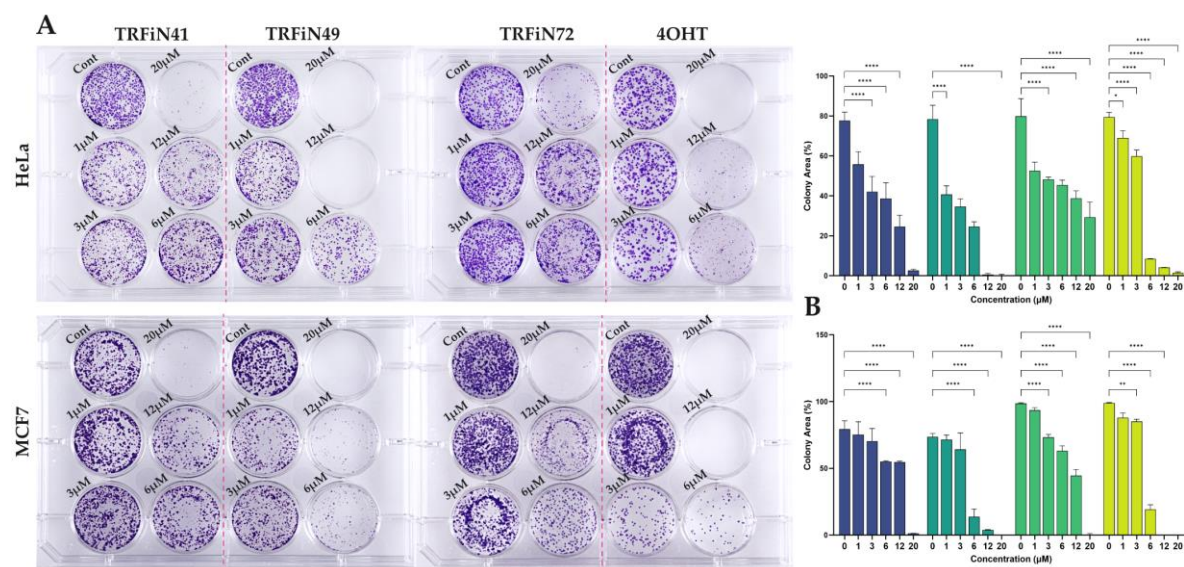


Figure 5.18. Effect of TRFiN compounds and 4-hydroxytamoxifen on colony formation in HeLa and MCF7 cells. (A) Representative colony formation images of HeLa and MCF7 cells after treatment with TRFiN41, TRFiN49, TRFiN72, and 4-hydroxytamoxifen at different concentrations. (B) Quantification of colony area in HeLa and MCF7 cells after treatment. Data represent mean $\pm$ SD ($n = 3$). Statistical analysis was conducted by two way ANOVA and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

The HeLa colony images in Figure 5.18A followed the quantified measurements shown in Figure 5.18B. The untreated wells contained dense and widely distributed crystal violet stained colonies, while treatment progressively reduced both the number and occupied area of the colonies. The loss of colony formation was already clear under TRFiN49 at intermediate concentrations, and almost no colonies were visible at 12 and 20 μM . A similar loss was present under 4OHT at the higher concentrations. TRFiN41 treated wells retained more colonies at the lower concentrations but showed a major reduction at 12 μM and very little staining at 20 μM . In contrast, TRFiN72 treated wells retained distinct colonies throughout the concentration range, including at 20 μM . The HeLa data therefore showed that the concentration required for marked suppression differed between the compounds, with TRFiN49 acting strongly at lower and intermediate concentrations, TRFiN41 becoming more active at the higher concentrations, and TRFiN72 retaining the greatest residual clonogenic growth. MCF7 cells also showed a concentration dependent loss of colony formation, but the response pattern differed from that observed in HeLa cells. At concentrations of 1 and 3 μM , TRFiN41 caused only a small reduction in colony area, whereas a clearer effect was observed at 6 and 12 μM . However, the reduction at 12 μM remained approximately 31%, showing that a substantial colony population was still present at this concentration. A sharp decline occurred at 20 μM , where TRFiN41 reduced the colony area by more than 98%.

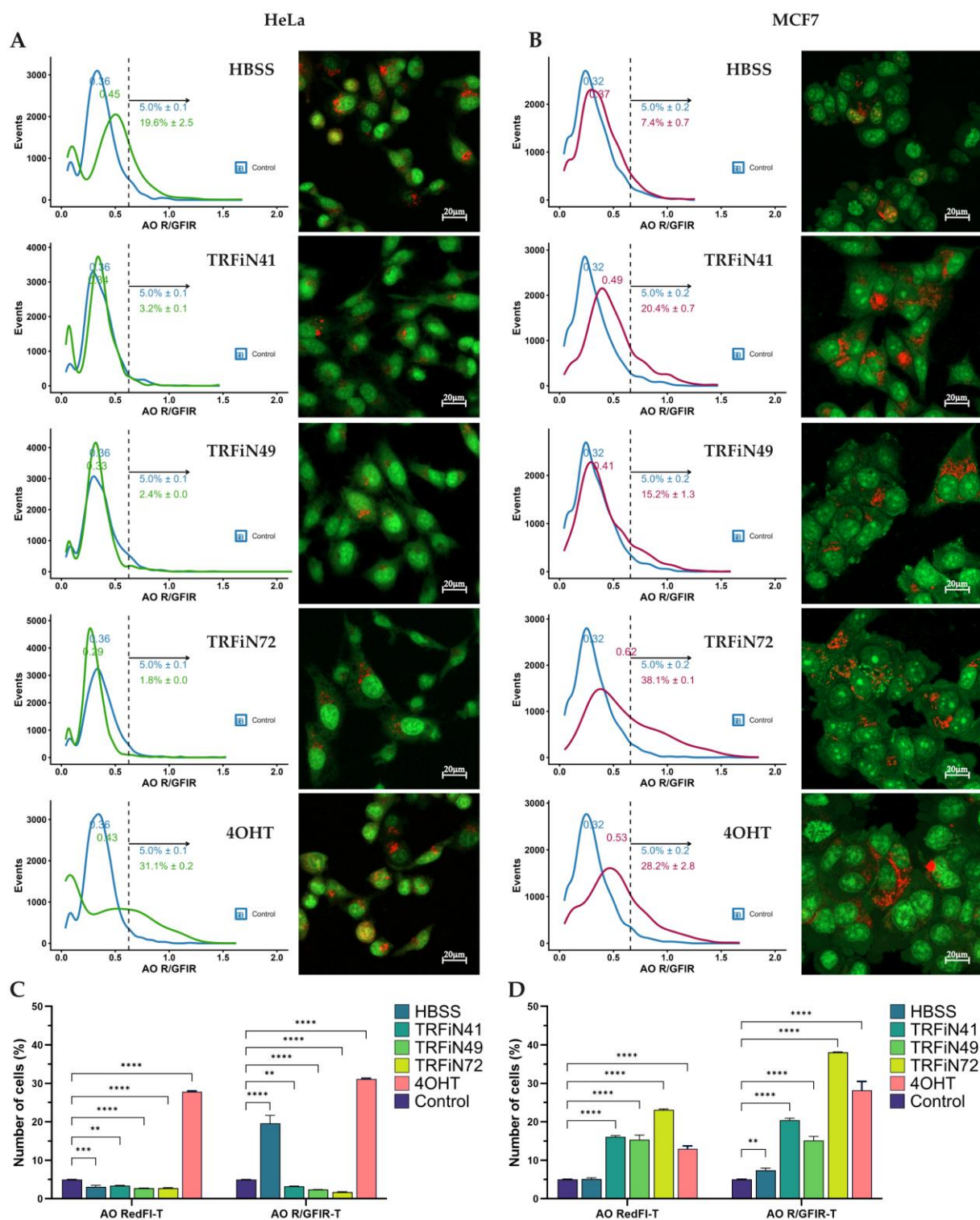


Figure 5.19. Effect of TRFiN compounds and 4-hydroxytamoxifen on acidic vesicular organelle accumulation in HeLa and MCF7 cells. (A,B) Representative acridine orange flow cytometry histograms and fluorescence microscopic images of HeLa and MCF7 cells, respectively, after treatment. (C,D) Quantification of acridine orange red fluorescence thresholding and acridine orange red to green fluorescence intensity ratio thresholding in HeLa and MCF7 cells, respectively. Data represent mean $\pm$ SD ($n = 3$). Statistical analysis was conducted by two way ANOVA and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

TRFiN72 followed a similar pattern but produced a somewhat greater effect at the intermediate concentrations. Its colony area was reduced by approximately one third at 6 μ M and by more than half at 12 μ M, followed by almost complete suppression at 20 μ M. The images in Figure 5.18A show that MCF7 colonies remained clearly visible under TRFiN41 and TRFiN72 at 6 and 12 μ M, whereas only minimal staining remained under either compound at the highest concentration. TRFiN49 and

4OHT produced an earlier and stronger reduction in MCF7 clonogenic growth. At 6 μM , both treatments reduced the colony area by approximately 81% relative to their respective controls. At 12 μM , TRFiN49 reduced colony formation by approximately 95%, while 4OHT almost completely removed the colony area. Only trace levels remained under either treatment at 20 μM . The quantified results in Figure 5.18B therefore show a clear separation between TRFiN49 and the other two TRFiN compounds at the intermediate concentrations. TRFiN49 caused a major loss of MCF7 colony formation from 6 μM onward, while TRFiN41 and TRFiN72 required the highest concentration to produce a comparable response. At 20 μM , however, all four treatments reduced colony formation by more than 98%, and the corresponding wells showed little or no visible crystal violet staining.

Acidic vesicular organelle accumulation was examined by acridine orange staining followed by flow cytometric and fluorescence microscopic analysis. The compounds were tested at their respective half maximal inhibitory concentration values, while cells incubated in Hank's balanced salt solution (HBSS) served as a starvation control. Acridine orange emits mainly green fluorescence when present throughout the cell but produces stronger red or orange fluorescence after accumulation in acidic compartments. Two measurements were used: the percentage of cells above the red fluorescence threshold and the percentage above the red to green fluorescence intensity ratio threshold. The ratio was calculated by dividing red acridine orange fluorescence by green fluorescence for each cell. This measurement allowed the increase in red acidic vesicle staining to be evaluated relative to the corresponding green cellular signal rather than relying on red fluorescence alone. HeLa cells showed little evidence of acidic vesicular organelle accumulation after treatment with the three TRFiN compounds. The untreated control remained low in both acridine orange measurements, while TRFiN41, TRFiN49, and TRFiN72 produced values at or below the control level. TRFiN41 caused a small reduction in both readouts, and still lower values were measured under TRFiN49 and TRFiN72. The histograms and fluorescence images in Figure 5.19A,C show that these treatment groups remained mainly green, with only a small number of red orange foci. The starvation control showed a different response depending on the measurement used. Red fluorescence thresholding decreased below the untreated control, but the red to green ratio increased by approximately fourfold. The ratio based distribution therefore detected a clear shift under starvation that was not identified by red fluorescence thresholding alone. The microscopic images also showed red orange vesicular staining in the HBSS treated cells. A much stronger HeLa response was observed with 4OHT. Red fluorescence thresholding increased by approximately 5.6-fold relative to control, while the red to green ratio increased by approximately 6.2-fold. The 4OHT histogram showed a marked shift away from the untreated distribution, and the fluorescence images in Figure 5.19A showed numerous red orange acidic structures within the treated cells. The response was greater than that observed under HBSS in both the ratio based measurement and the microscopic staining. In contrast, the three TRFiN treated groups retained predominantly green fluorescence and remained below the control in the quantified data presented in Figure 5.19C.

MCF7 cells showed a different acridine orange response, with increases under all three TRFiN compounds. TRFiN41 and TRFiN49 each increased red fluorescence thresholding by slightly more than threefold relative to the control. The red to green ratio also increased by approximately fourfold under TRFiN41 and threefold under TRFiN49. TRFiN72 produced the largest change among the tested compounds, increasing red fluorescence by approximately 4.6-fold and the red to green ratio by approximately 7.6-fold. Treatment with 4OHT also produced a clear response, with an approximately 2.6-fold increase in red fluorescence and a 5.6-fold increase in the ratio. The HBSS control produced little change in red fluorescence and a smaller increase in the ratio than the compound treated groups. The flow cytometric distributions in Figure 5.19B showed clear shifts towards stronger red orange fluorescence in the treated MCF7 populations, particularly under TRFiN72 and 4OHT. The fluorescence microscopy images in the same panel showed mainly green staining with few red structures in the untreated control, whereas the treated cells contained numerous red orange foci distributed throughout the cytoplasm. This vesicular staining was most extensive under TRFiN72 and

4OHT, followed by TRFiN41 and TRFiN49. The quantified values in Figure 5.19D followed the same order, with TRFiN72 producing the highest ratio based response among the treatment groups.

5.15 TRFiN compounds inhibit chorioallantoic membrane angiogenesis and reduce *in ovo* microtumour growth without evident organ toxicity

The chorioallantoic membrane (CAM) assay was used to examine the effects of TRFiN41, TRFiN49, TRFiN72, and the parent compound A822 on vascular branching. Chicken embryos were used for the angiogenesis experiment because the broad vascular area of the chorioallantoic membrane allowed changes in the developing vessel network to be followed over time in the *ex ovo* assay. The compounds were tested at concentrations ranging from 5 to 20 μM , and vascular branching was measured at 6, 24, and 48 h. Representative images of the treated membranes are presented in Figure 5.20A–D, while the corresponding branching point measurements are shown in Figure 5.20E–H. A preliminary concentration assessment identified 230 μM as the maximum lethal dose for the compounds. The concentrations used for the angiogenesis experiment were therefore substantially below this level. At 6 h, the vascular networks surrounding the treatment discs remained similar to the corresponding controls. The branching point analysis showed some variation between concentrations, but none of the compounds produced a consistent reduction at this early time point.

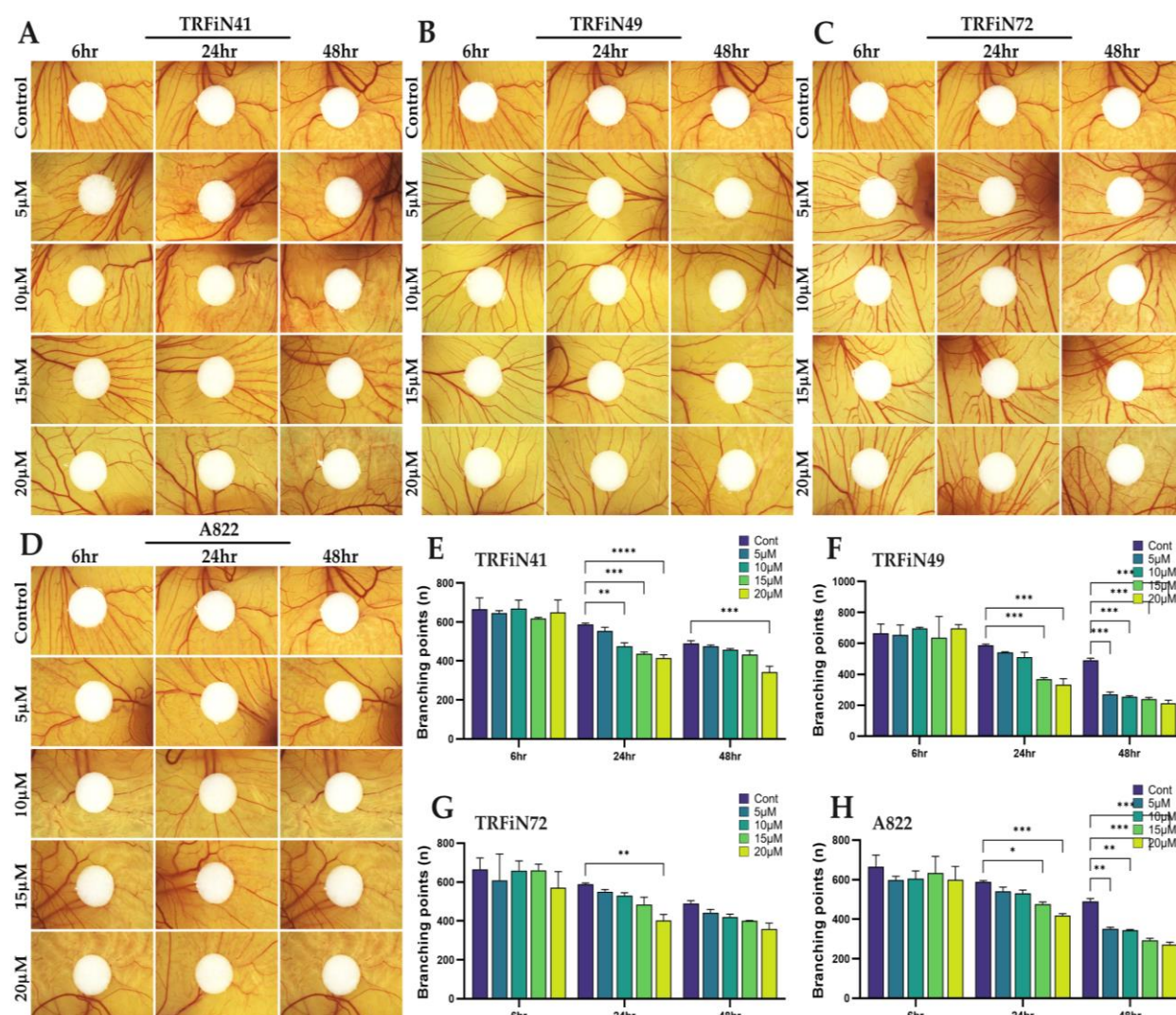


Figure 5.20. Effect of TRFiN compounds and A822 on chorioallantoic membrane angiogenesis. (A,D) Representative images of vascular branching after treatment with TRFiN41, TRFiN49, TRFiN72, and A822, respectively, at 6, 24, and 48 h. (E–H) Quantification of branching points after treatment with TRFiN41, TRFiN49, TRFiN72, and A822, respectively, at different concentrations. Data represent mean $\pm$ SD ($n = 4$). Statistical analysis was conducted by two way ANOVA and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

The control membranes retained a dense network of vessels around the disc, and the treated membranes showed a comparable arrangement in the representative images in Figure 5.20A-D. Differences became clearer after 24 h, when the higher compound concentrations reduced the formation of new branching points. The reduction became more pronounced at 48 h, and the membranes treated at 15 and 20 μM showed visibly simpler vascular networks than the controls. The existing major vessels remained visible, while the number of smaller branches around the treatment area decreased. TRFiN41 produced a moderate concentration dependent reduction in vascular branching. At 5 μM , its effect remained small at both 24 and 48 h, whereas clearer reductions were recorded at 10 and 15 μM . At the highest tested concentration, the number of branching points was approximately 29% below the control at 24 h and 30% below the control at 48 h. The images in Figure 5.20A show that the vascular network around the TRFiN41 treated discs became less dense as the concentration increased, particularly during the later observation periods. This pattern was also present in the quantified branching point measurements in Figure 5.20E, where the greatest separation from the control was recorded at 20 μM .

TRFiN49 produced the greatest reduction in vascular branching among the tested compounds. Its early measurements remained close to the control, but a clear concentration related effect appeared at 24 h. At this time point, the two lower concentrations caused only moderate changes, while treatment at 15 and 20 μM markedly reduced the number of vessel branches. The highest concentration produced an approximately 44% reduction relative to the control. The effect increased further at 48 h, when all four concentrations produced fewer branching points than the untreated membrane. At 20 μM , branching was reduced by approximately 56%, and the corresponding membrane showed a much less developed vascular network in Figure 5.20B. The measurements in Figure 5.20F also show that TRFiN49 caused a strong response even at the lower concentrations during the final observation period. TRFiN72 reduced vascular branching more moderately than TRFiN41 and TRFiN49. Only limited changes were present at 5 and 10 μM , while the effect became clearer at the two higher concentrations. At 20 μM , branching was approximately 32% below the control at 24 h and approximately 27% below the control at 48 h. The representative membranes in Figure 5.20C retained more visible secondary branches than those treated with the corresponding concentrations of TRFiN49. The quantified results in Figure 5.20G nevertheless showed a gradual reduction as the TRFiN72 concentration increased, with the lowest branching point number recorded under the 20 μM treatment. The parent compound A822 also caused a clear concentration dependent reduction in vascular development. The branching pattern remained close to the control at 6 h but separated progressively at the later time points. At 24 h, the effect was most visible at 15 and 20 μM . By 48 h, all A822 concentrations had reduced branching, and the greatest decrease was recorded at 20 μM , where the number of branching points was approximately 45% below the control. The vessel network in Figure 5.20D became increasingly sparse around the treatment disc as the concentration increased, and the same pattern was present in the measurements in Figure 5.20H. At the final time point and highest concentration, TRFiN49 produced the greatest reduction, followed by A822, TRFiN41, and TRFiN72.

The effects of the compounds on tumour growth were examined using HeLa and MCF7 microtumours established on the chorioallantoic membrane of quail embryos. The cells were deposited in Matrigel and allowed to form localised microtumours before treatment. A concentration of 20 μM was selected for this experiment based on the preliminary embryo viability assessment. Following treatment, the tumours were removed from the membrane and weighed. Representative microtumours and their quantified weights are presented in Figure 5.21A,B, while the embryo preparation and arrangement of the *ex ovo* system are shown in Figure 5.21C,D. All four compounds reduced HeLa microtumour weight. The untreated tumours had a mean weight of approximately 193 mg, whereas the treated groups ranged from approximately 104 to 160 mg. TRFiN41 produced the smallest reduction, lowering tumour weight by approximately 17%. A greater effect was observed under TRFiN49, which reduced the weight by approximately 36%.

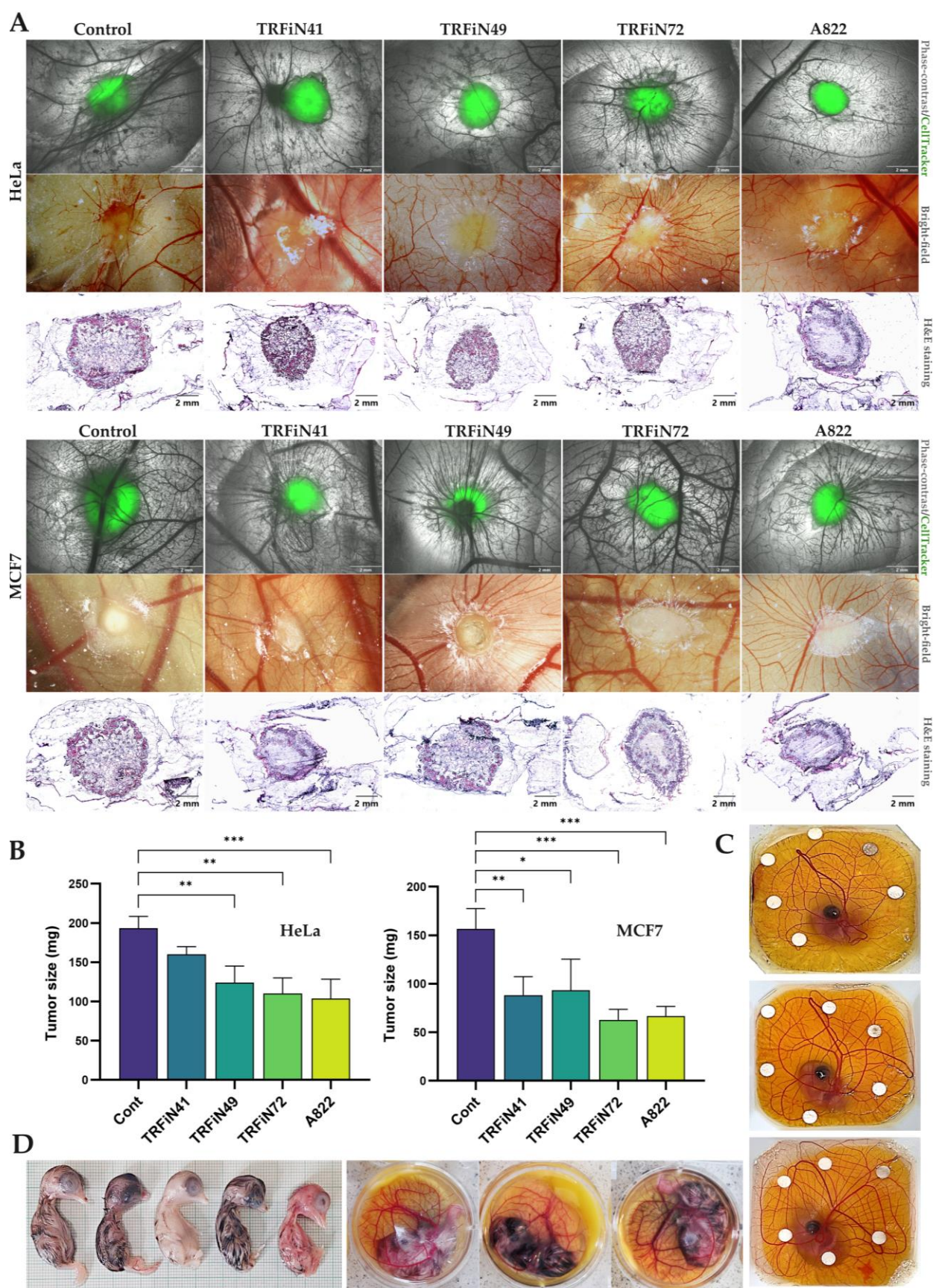


Figure 5.21. Effect of TRFiN compounds and A822 on *in ovo* microtumour growth. (A) Representative images of HeLa and MCF7 microtumours after treatment. (B) Quantification of HeLa and MCF7 microtumour size after treatment. (C) Representative image of the angiogenesis setup (*ex ovo*). (D) Representative images of quail embryos and embryos placed in a well of six well plates. Data represent mean $\pm$ SD ($n = 4$). Statistical analysis was conducted by two way ANOVA and post hoc Dunnett's test. $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, and $P \leq 0.00001$.

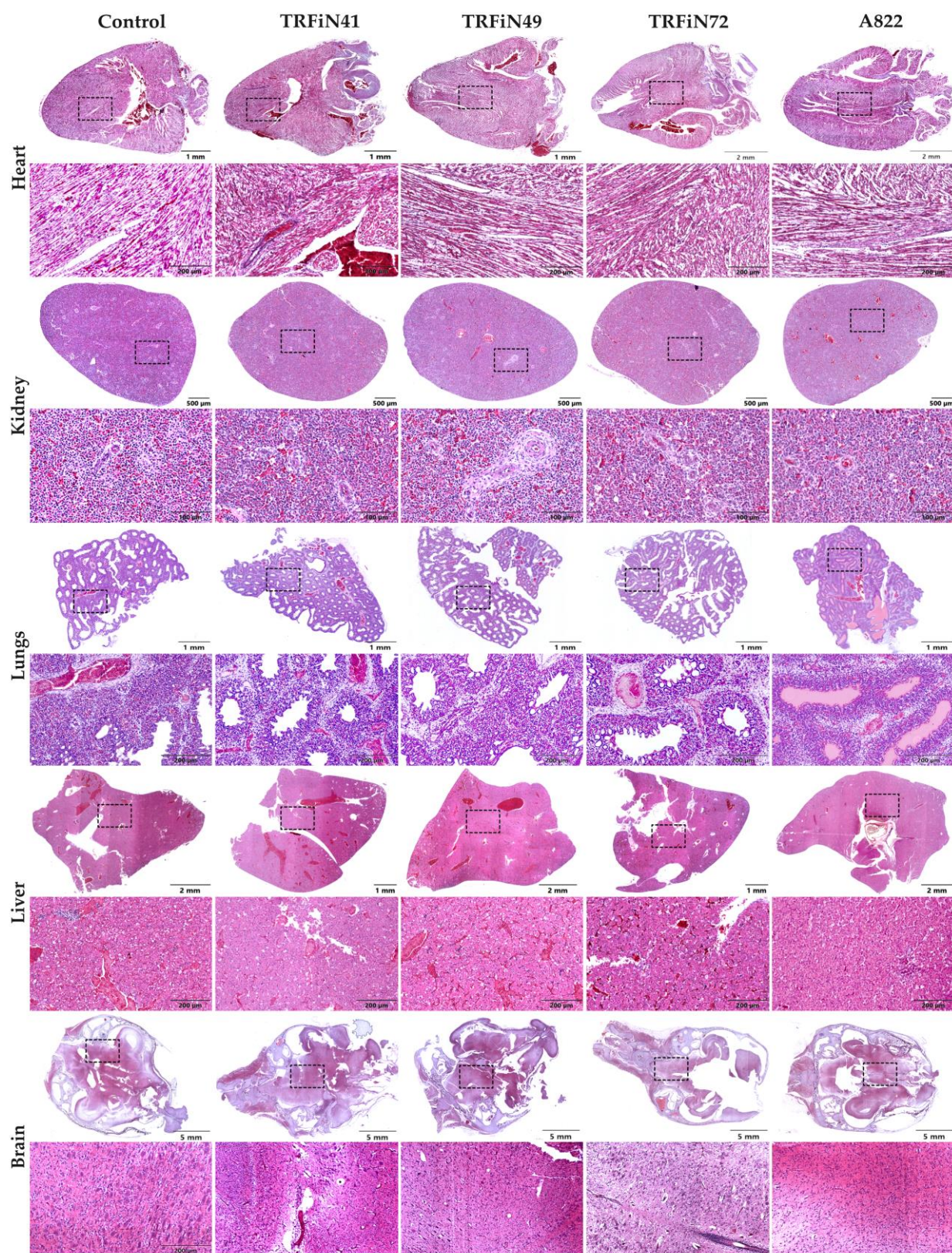


Figure 5.22. *Histological evaluation of quail embryo organs after treatment with TRFiN compounds and A822.* Haematoxylin and eosin staining of chicken embryo heart, kidney, lungs, liver, and brain sections after treatment with TRFiN41, TRFiN49, TRFiN72, and A822, compared with the untreated control. For each organ, low magnification images show the overall tissue architecture, and the boxed regions indicate the areas selected for higher magnification observation. The enlarged fields show the cellular organisation and tissue morphology of each organ after compound exposure. Scale bars are indicated in each panel.

TRFiN72 and A822 produced the largest decreases, reducing HeLa microtumour weight by approximately 43% and 46%, respectively. These differences were visible in the excised tumour images in Figure 5.21A, where the A822- and TRFiN72 treated tumours appeared smaller than the control and TRFiN41 treated tumours. The quantified measurements in Figure 5.21B showed the lowest mean HeLa tumour weight under A822, followed closely by TRFiN72. The reduction in MCF7 microtumour weight was stronger than that observed in HeLa tumours under several of the treatments. The untreated MCF7 tumours had a mean weight of approximately 157 mg. TRFiN41 and TRFiN49 reduced the tumour weight by approximately 44% and 40%, respectively. TRFiN72 produced the greatest reduction, lowering the weight by approximately 60%, while A822 produced a similar decrease of approximately 57%. The representative images in Figure 5.21A show smaller MCF7 tumour masses in all treatment groups, with the smallest tumours observed after TRFiN72 and A822 exposure. The quantified results in Figure 5.21B followed the same pattern, although greater variation between replicates was recorded under TRFiN49 than under the other treatments.

The microtumours remained localised within the membrane implantation area during the treatment period. The images in Figure 5.21C,D show the arrangement of the *ex ovo* preparation, the position of the embryo within the culture well, and the vascular membrane surrounding the implanted tumour. The gross appearance of the embryos did not show clear differences between the untreated and treated groups at the tested concentration. Histological examination was carried out on heart, kidney, lungs, liver, and brain sections collected from treated chicken embryos. Low magnification views and enlarged areas of the haematoxylin and eosin stained tissues are presented in Figure 5.22. The myocardial arrangement of the heart remained similar across the control and treatment groups, without visible disruption of the general tissue structure. Kidney sections retained the normal organisation of the developing renal tissue, and no clear treatment related damage was visible in the examined fields. The lung sections showed a comparable parenchymal appearance across the groups, while the liver sections retained their general cellular and tissue organisation after exposure to TRFiN41, TRFiN49, TRFiN72, and A822. Brain sections also showed similar overall morphology in the control and treated embryos. No clear areas of tissue breakdown, widespread cell loss, or major structural alteration were visible in the examined organs at 20 μ M.

5.16 TRFiN compounds increase telomere aberrations with only limited reduction in telomere fluorescence intensity

Telomere related changes were examined using metaphase telomere fluorescence *in situ* hybridisation, Flow-FISH, and colocalisation analysis of TIN2 with TRF1/2. Flow-FISH was used to measure changes in the telomere fluorescence signal across the cell population, while metaphase telomere fluorescence *in situ* hybridisation allowed individual chromosome ends and visible telomere abnormalities to be examined. The Flow-FISH histograms and quantified fluorescence loss measurements for HeLa and MCF7 cells are presented in Figure 5.23B,C. In both cell lines, treatment produced only a limited shift in the telomere fluorescence distribution compared with the untreated control. In HeLa cells, the mean fluorescence loss was approximately 7% in the control and increased to between 9% and 12% after treatment. TRFiN72 produced the largest change, followed by TRFiN41 and A822, while TRFiN49 produced the smallest increase. These treated values were approximately 1.3- to 1.7-fold above the control, and the corresponding histograms showed only a small displacement of the fluorescence peaks rather than a large separation between the untreated and treated populations.

MCF7 cells showed slightly greater telomere fluorescence loss than HeLa cells, although the changes remained moderate. The untreated MCF7 population showed approximately 10% fluorescence loss, while the treated groups ranged from approximately 16% to 17%. TRFiN72 again produced the highest mean value, while TRFiN41 and TRFiN49 produced similar responses and A822 produced a slightly smaller change. These values represented increases of approximately 1.5- to 1.7-fold relative to the control. The MCF7 Flow-FISH distributions in Figure 5.23B,C remained close

to one another, with no large movement of the treated peaks away from the control. The treatment related changes in bulk fluorescence were therefore limited in both cell lines during the examined period.

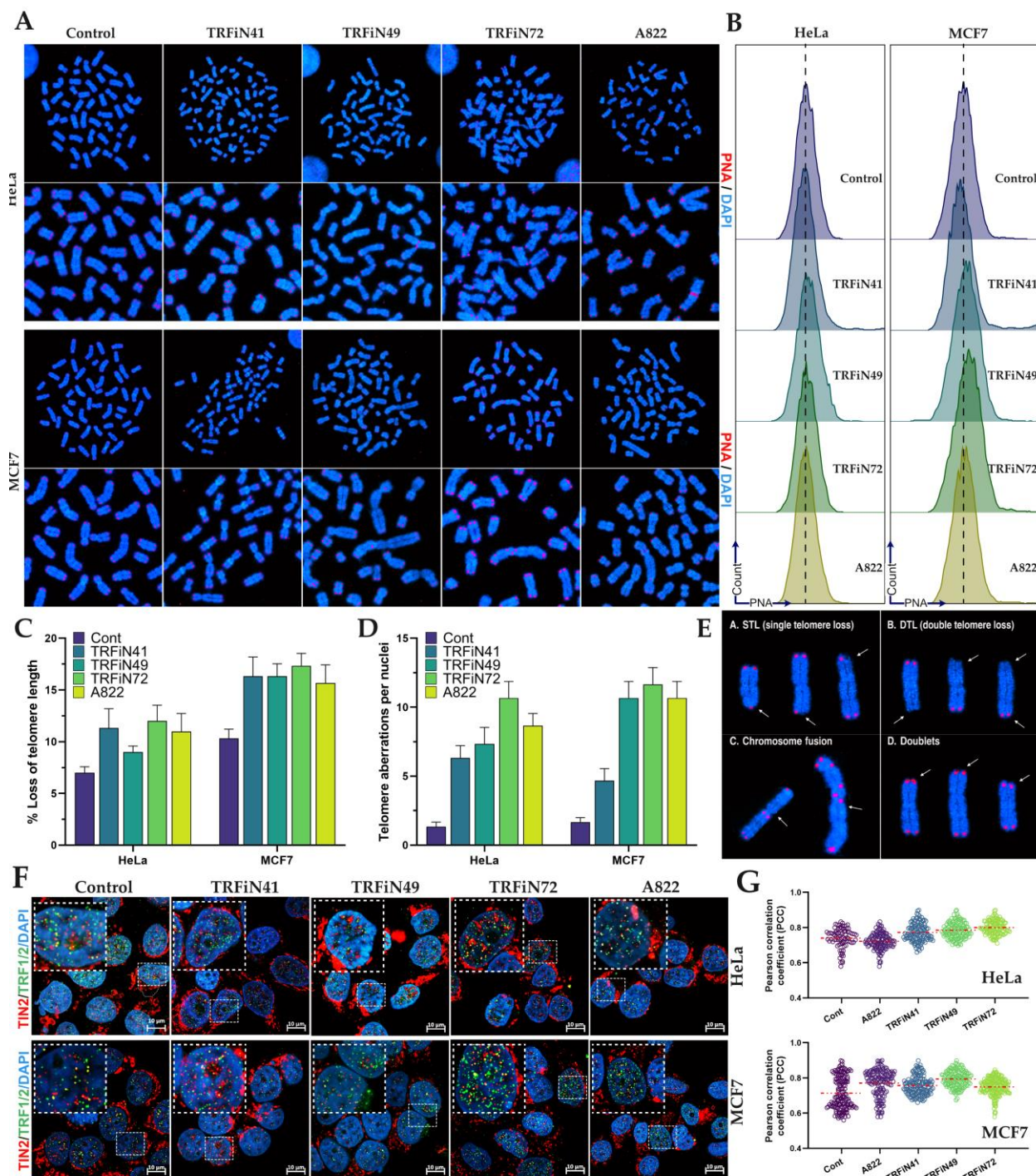


Figure 5.23. Effect of TRFiN compounds and A822 on telomere integrity and TIN2/TRF1/2 colocalisation in HeLa and MCF7 cells. (A) Representative metaphase telomere fluorescence *in situ* hybridisation images of HeLa and MCF7 cells after treatment. (B,C) Flow fluorescence *in situ* hybridisation histograms and quantification of telomere fluorescence loss in HeLa and MCF7 cells. (D) Average telomere aberrations per nucleus in HeLa and MCF7 cells after treatment. (E) Representative images of major telomere aberrations, including single telomere loss, double telomere loss, chromosome fusion, and doublets. (F,G) Representative colocalisation images of TIN2 and TRF1/2 and quantification of Pearson correlation coefficient in HeLa and MCF7 cells. Data represent mean $\pm$ standard deviation ($n = 3$). Statistical analysis was conducted by one way analysis of variance and post hoc Dunnett's test.

More pronounced changes were detected when individual chromosome ends were examined in metaphase preparations. Representative telomere fluorescence *in situ* hybridisation images are shown in Figure 5.23A, and the quantified numbers of telomere aberrations per nucleus are presented in Figure 5.23D. Untreated HeLa cells contained a low mean number of abnormalities, with approximately one telomere aberration per nucleus. All four treatments increased this value, although the degree of increase differed between the compounds. TRFiN41 and TRFiN49 increased the mean aberration count to approximately fivefold above the control, while A822 produced an increase of more than sixfold. TRFiN72 produced the strongest response in HeLa cells, with the mean number of aberrations increasing to approximately eightfold above the untreated level. The metaphase images showed that telomere signals remained present on most chromosomes, but treated cells contained a greater number of chromosome ends with missing, duplicated, or irregular telomere signals. MCF7 cells also showed a low number of telomere abnormalities under the untreated condition and a clear increase following treatment. TRFiN41 produced the smallest response, increasing the mean number of aberrations to approximately threefold above the control. Much larger increases were recorded under TRFiN49, TRFiN72, and A822. Each of these treatments produced more than a sixfold increase, with TRFiN72 giving the highest mean aberration count. The quantified measurements in Figure 5.23D showed that the responses to TRFiN49 and A822 were nearly identical in MCF7 cells, while the TRFiN72 treated group showed a slightly greater value. The increase in MCF7 telomere abnormalities under these three treatments was therefore greater than that observed under TRFiN41.

Several forms of telomere abnormality were visible in the treated metaphase preparations. Representative examples of single telomere loss, double telomere loss, chromosome fusion, and telomere doublets are presented in Figure 5.23E. Single telomere loss appeared as the absence of a detectable telomere signal from one chromosome end, while double telomere loss involved loss of signal from both corresponding ends. Telomere doublets appeared as split or duplicated signals at a chromosome end rather than a single compact signal. Chromosome fusions were visible as joined chromosome ends without a normal separation between the chromosomes. These abnormalities occurred in both HeLa and MCF7 cells and were observed more frequently in the treated metaphase spreads than in the corresponding controls. The spatial association of TIN2 with TRF1/2 was examined by immunofluorescence colocalisation analysis. Representative nuclear staining and the quantified Pearson correlation coefficients are shown in Figure 5.23F,G. In untreated HeLa cells, TIN2 and TRF1/2 signals showed a high degree of overlap, with a mean Pearson correlation coefficient (PCC) of approximately 0.74. The treated groups remained within a similar range, extending from approximately 0.72 under A822 to 0.80 under TRFiN72. TRFiN41 and TRFiN49 produced intermediate values. The representative images showed overlapping TIN2 and TRF1/2 signals within the nuclei under both control and treatment conditions, without a marked loss of the overlapping fluorescent regions. A similar pattern was observed in MCF7 cells. The untreated population had a mean PCC of approximately 0.71, while the treated groups ranged from approximately 0.75 to 0.79. TRFiN49 produced the highest mean coefficient, followed by A822 and TRFiN41, while TRFiN72 produced the lowest treated value. All four treated values remained close to the untreated range, and the nuclear images in Figure 5.23F,G continued to show overlapping TIN2 and TRF1/2 signals after treatment. No major spatial separation of the two staining patterns was detected in either cell line.

5.17 TRFiN compounds induce protein changes consistent with DNA damage response, cell cycle suppression, senescence, and apoptotic signalling

Western blot analysis was performed to examine protein level changes after treatment with TRFiN41, TRFiN49, TRFiN72, and A822 in MCF7 and HeLa cells. Beta-actin was used as the loading control, and the band intensities were normalized to beta-actin and expressed as fold change relative to the untreated control, which was set to 1. Full length blots are provided in the Supplementary File.

Among the proteins related to senescence and cell cycle regulation, Lamin B1 decreased in both MCF7 and HeLa cells after treatment. The reduction was moderate in MCF7 cells, where Lamin B1 was generally reduced by approximately 20-60%, while a stronger reduction of approximately 50-70% was observed in HeLa cells. Loss of Lamin B1 is commonly associated with cellular senescence, and this reduction agrees with the senescence phenotype observed in the earlier experiments. p21 showed only limited changes under most treatment conditions. In MCF7 cells, p21 remained close to the control after TRFiN41, TRFiN49, and TRFiN72 treatment, while A822 caused a moderate reduction. A similar pattern was observed in HeLa cells, although A822 produced a stronger decrease, reducing p21 to less than one third of the control level. In contrast, p27 Kip1 increased consistently in both cell lines. The increase was approximately 1.7-1.9-fold in MCF7 cells and approximately 2.0-2.6-fold in HeLa cells. This increase in p27 Kip1, together with the other changes in cell cycle proteins, is consistent with reduced cell cycle progression following treatment (Figure 5.24A,B).

Cyclin E1 decreased in both cell lines, with the reduction being stronger in HeLa cells. In MCF7 cells, Cyclin E1 was reduced by approximately 30-40% under TRFiN41, TRFiN49, and TRFiN72, while A822 remained close to the control. In HeLa cells, Cyclin E1 was reduced by approximately 45-85%. Cyclin D1 showed the clearest difference between the two cell lines. In MCF7 cells, Cyclin D1 increased very strongly under all four treatments, reaching approximately 22-25-fold above the control. In HeLa cells, however, Cyclin D1 decreased by approximately 55-70% after TRFiN41, TRFiN49, and TRFiN72 treatment, while A822 increased it by approximately 2-fold. p16 was not detected in MCF7 cells, whereas in HeLa cells it increased to approximately 1.5-1.9-fold of the control level after treatment. These differences show that the two cell lines responded differently at the level of cell cycle control. The strongest treatment related changes were observed in proteins involved in DNA damage and checkpoint signalling. γ H2AX increased clearly in both cell lines, indicating the presence of DNA damage after treatment. In MCF7 cells, γ H2AX increased by approximately 3-4-fold, while in HeLa cells it increased by approximately 6-7-fold. The stronger γ H2AX response in HeLa cells was accompanied particularly by the marked pCHK2/CHK2 increase described below.

The pCHK1/CHK1 ratio increased in both cell lines but was more pronounced in MCF7 cells. In MCF7 cells, the ratio increased approximately 7-10-fold compared with the control, whereas in HeLa cells it increased approximately 2.5-3.5-fold. Total CHK1 decreased in both cell lines, particularly in MCF7 cells. The pATM/ATM ratio also increased strongly, reaching approximately 3-4-fold in MCF7 cells and approximately 4-6-fold in HeLa cells. Similarly, the pATR/ATR ratio increased by approximately 3-4-fold in MCF7 cells and approximately 2-2.6-fold in HeLa cells. In comparison, changes in total ATM and ATR were smaller than the changes in the corresponding phosphorylated to total ratios. A marked difference between the two cell lines was observed for pCHK2/CHK2. The phosphorylated CHK2 signal was not detected in MCF7 cells, whereas the pCHK2/CHK2 ratio increased very strongly in HeLa cells, reaching approximately 22-28-fold above the control. Total CHK2 remained close to the control in both cell lines. These results show that both cell lines activated DNA damage checkpoints, but the relative contribution of the checkpoint pathways differed. MCF7 cells showed a stronger pCHK1/CHK1 response, whereas HeLa cells showed a very strong pCHK2/CHK2 response together with a larger increase in γ H2AX. The combined changes in γ H2AX, pCHK1/CHK1, pCHK2/CHK2, pATM/ATM, and pATR/ATR support activation of the DNA damage response following treatment (Figure 5.24A,B).

Proteins related to apoptosis also showed treatment dependent changes. Bcl-2, an anti-apoptotic protein, decreased in both cell lines and was generally reduced to approximately half of the control level. In contrast, BAX increased strongly in MCF7 cells, reaching approximately 3-fold above the control after treatment. In HeLa cells, BAX remained close to the control under TRFiN72 and increased more modestly under TRFiN41, TRFiN49, and A822, reaching up to approximately 1.6-fold. PUMA also increased in both cell lines, reaching approximately 2-fold above the control in most treatment groups. The decrease in Bcl-2 together with the increase in PUMA and the stronger BAX increase in MCF7 cells supports a shift toward pro-apoptotic signalling. Cytochrome c remained close to the

control in both cell lines, indicating no large change in the total detected cytochrome c protein level. AIF also showed only small changes in most treatment groups, although some reduction was observed under TRFiN72 and A822, particularly in HeLa cells. PARP remained close to the control in MCF7 cells. In HeLa cells, the full length PARP signal showed a moderate reduction, particularly after A822 treatment, and lower PARP bands were visible in the treated lanes. This pattern is consistent with PARP cleavage during apoptosis and agrees with the apoptotic response observed in the earlier experiments.

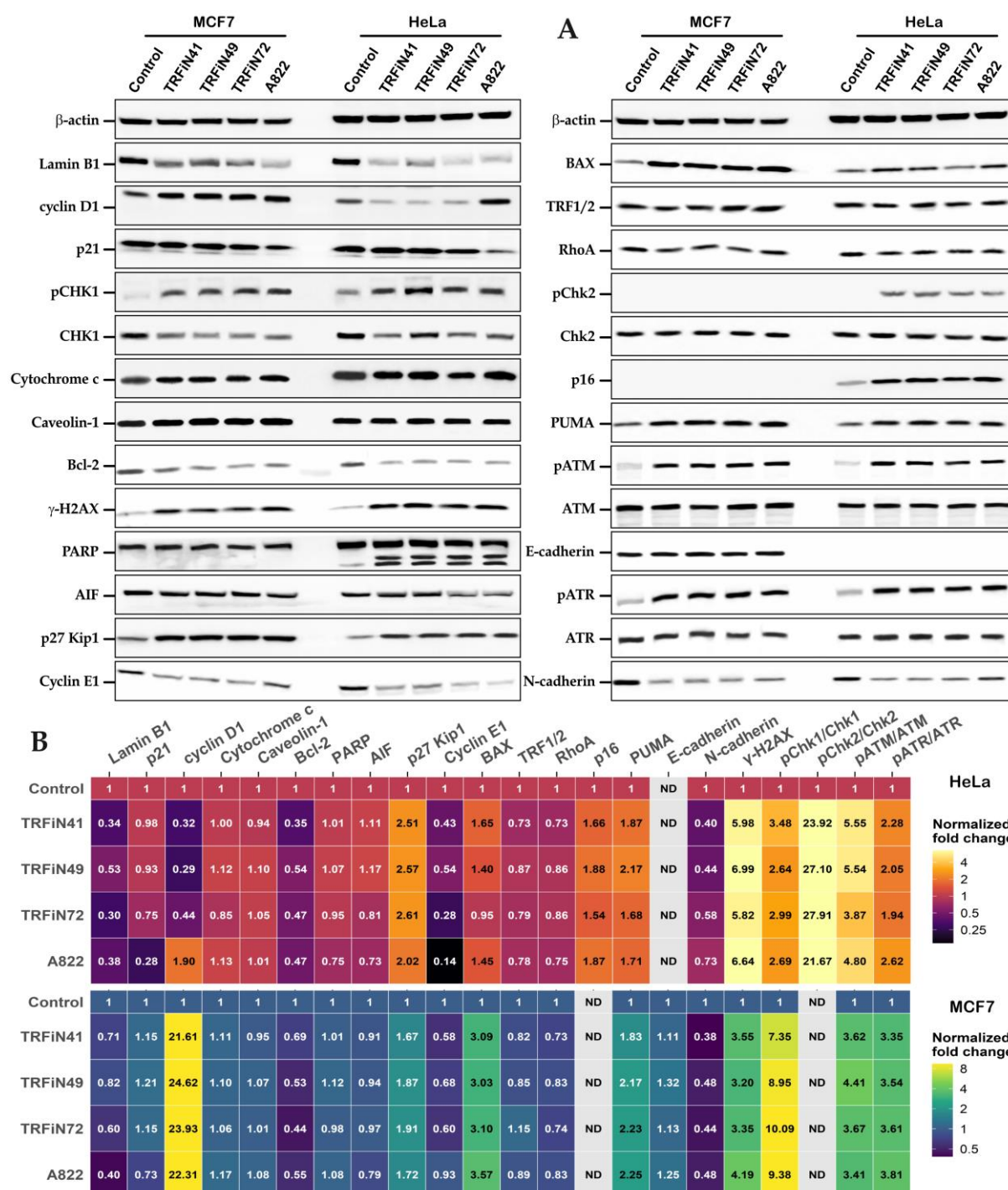


Figure 5.24. Effect of TRFiN compounds and A822 on protein expression in MCF7 and HeLa cells. (A) Representative western blot bands of proteins related to senescence, cell cycle regulation, DNA damage response, apoptosis, migration, and telomere associated signalling in MCF7 and HeLa cells after treatment with TRFiN41, TRFiN49, TRFiN72, and A822. (B) Heat map showing protein fold changes in MCF7 and HeLa cells, normalized to beta-actin and expressed relative to the untreated control (set to 1). Phosphorylated checkpoint proteins are shown as phospho/total protein ratios. Full length blots are provided in the Supplementary File.

Proteins related to migration, adhesion, and telomere associated structure showed smaller changes. Caveolin-1 remained close to the control in both cell lines. RhoA showed a small reduction, while the combined TRF1/2 signal remained relatively close to the control, with no large treatment related loss in the measured signal. E-cadherin was detected in MCF7 cells and remained close to, or slightly above, the control level, whereas it was not detected in HeLa cells. N-cadherin decreased in both cell lines, by approximately 50-60% in MCF7 cells and approximately 25-60% in HeLa cells. This reduction agrees with the earlier migration experiments, where treatment reduced wound healing and cell front velocity (Figure 5.24A,B).

5.18 Pathway level transcriptomic profiling of TRFiN41, TRFiN49, TRFiN72 and A822 in MCF7 and HeLa cells

To examine how prolonged telomere directed treatment altered global cellular programmes and functions, comparative transcriptome analysis was performed in HeLa and MCF7 cells after 168 h of exposure to TRFiN41, TRFiN49, TRFiN72, and the parent compound A822 at their respective half maximal inhibitory concentrations. The detailed script and the high clarity diagram for this next generation sequencing (NGS) transcriptomic analysis are provided in the flash drive attached to the back cover of the thesis. Differential expression and pathway enrichment were carried out within the R based workflow described in the Materials and Methods chapter. Particular attention was given to pathways related to DNA replication, cell cycle progression, senescence, and apoptosis, since these processes were expected to change after long term telomere disruption.

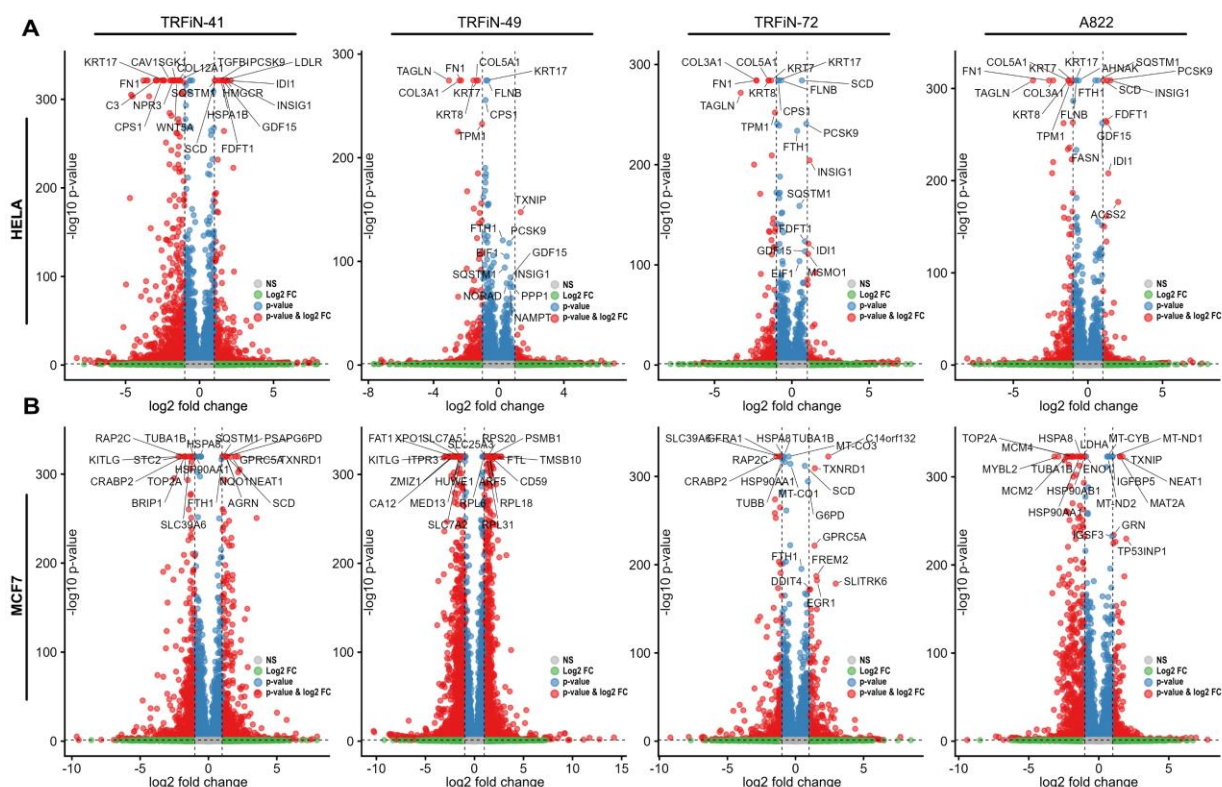


Figure 5.25. Differential gene expression profiles after 168 h treatment with TRFiN compounds and A822 in HeLa and MCF7 cells. (A) Volcano plots of HeLa cells for TRFiN41, TRFiN49, TRFiN72, and A822 versus control. (B) Volcano plots of MCF7 cells for TRFiN41, TRFiN49, TRFiN72, and A822 versus control. Genes are distributed according to log2 fold change and $-\log_{10}$ P value.

The first clear difference was the scale of the transcriptional response in the two cell lines. In HeLa cells, TRFiN41 produced the broadest response, with 1500 differentially expressed genes, including 499 upregulated and 1001 downregulated transcripts. A822 produced 502 differentially expressed genes, while TRFiN49 and TRFiN72 showed narrower responses, with 421 and 366 genes, respectively. In MCF7 cells, the response was generally much stronger. TRFiN49 produced the largest shift, with

3906 differentially expressed genes, including 1771 upregulated and 2135 downregulated transcripts. TRFiN41 also caused a broad response, with 1623 genes, followed by A822 with 1170 and TRFiN72 with 905. The volcano plots reflect this difference well. In HeLa cells, the widest distribution was seen under TRFiN41, whereas in MCF7 cells the most extensive spread was seen under TRFiN49, with a much denser set of significant transcripts on both sides of the plot (Figure 5.25). The HeLa expression profile was dominated by suppression of extracellular matrix, focal adhesion, and cytoskeletal programmes rather than by strong repression of the core DNA replication machinery. Across all four HeLa comparisons, the main suppressed pathways repeatedly included cytoskeleton in muscle cells, focal adhesion, integrin signalling, extracellular matrix receptor interaction, and phosphatidylinositol 3-kinase/protein kinase B signalling. This common pattern was present under TRFiN41, TRFiN49, TRFiN72, and A822, which indicates a shared response in this cell line. The circos plots showed that genes contributing to these pathways were distributed across many chromosomes rather than being restricted to one locus, which is more consistent with coordinated transcriptional reprogramming than with a focal genomic effect (Figure 5.26A-D). The volcano plots support this interpretation at gene level. Recurrently altered transcripts included fibronectin 1, collagen type III alpha 1 chain, collagen type V alpha 1 chain, transgelin, tropomyosin 1, keratin 8, and keratin 17, all of which fit with the suppression of matrix and cytoskeletal organisation seen in the pathway analysis.

The activated pathway pattern in HeLa cells was more compound specific. TRFiN41 and A822 showed enrichment of sterol and lipid related pathways, including steroid biosynthesis and terpenoid backbone biosynthesis, while A822 also included cholesterol metabolism and peroxisome proliferator activated receptor signalling among the strongest activated pathways. This agrees with the appearance of sterol related genes such as farnesyl diphosphate farnesyltransferase 1, insulin induced gene 1, isopentenyl diphosphate delta isomerase 1, and proprotein convertase subtilisin/kexin type 9 among the prominent altered transcripts. TRFiN49 produced a different activated profile in HeLa cells, with circadian rhythm, cytokine cytokine receptor interaction, Janus kinase signal transducer and activator of transcription signalling, Notch signalling, and phospholipase D signalling represented among the top enriched pathways. TRFiN72 showed only a limited activated profile, with neuroactive ligand receptor interaction present in the top list. The HeLa transcriptome therefore showed a strong common loss of adhesion and structural programmes, together with compound specific changes in sterol metabolism and signalling. The MCF7 profile was different and showed a much stronger connection to the preselected pathways related to proliferation and genome maintenance. TRFiN41, TRFiN72, and A822 all showed suppression of cell cycle and DNA replication among the strongest enriched terms. Under TRFiN41, the suppressed pathways included cell cycle, DNA replication, homologous recombination, Fanconi anaemia pathway, mismatch repair, base excision repair, nucleotide metabolism, pyrimidine metabolism, and p53 signalling. Under TRFiN72, the suppressed list again included cell cycle, Fanconi anaemia pathway, and DNA replication. A822 produced a similar pattern, with suppression of cell cycle, DNA replication, homologous recombination, base excision repair, Fanconi anaemia pathway, mismatch repair, and p53 signalling. These enrichments place the MCF7 response directly within the framework of impaired replication, checkpoint engagement, and reduced proliferative capacity. The circos and bubble plots showed that these genes were distributed across multiple chromosomes and contributed to a broad pathway level response rather than a narrow gene cluster effect (Figure 5.26E-H).

The MCF7 TRFiN49 profile stood apart from the other treatments. It produced the largest number of differentially expressed genes and showed a broader metabolic and stress associated pathway pattern. The activated pathways included oxidative phosphorylation, chemical carcinogenesis reactive oxygen species, thermogenesis, and several neurodegeneration associated terms that commonly appear when mitochondrial and respiratory genes are strongly represented. The suppressed side included cell cycle, endocrine resistance, and cellular senescence. This suggests that in MCF7 cells TRFiN49 did not simply reduce cell cycle genes, but induced a wider metabolic and stress linked transcriptional response. The volcano plot is consistent with this scale of change, because TRFiN49

produced the densest and widest set of significant transcripts among the MCF7 treatments (Figure 5.25B).

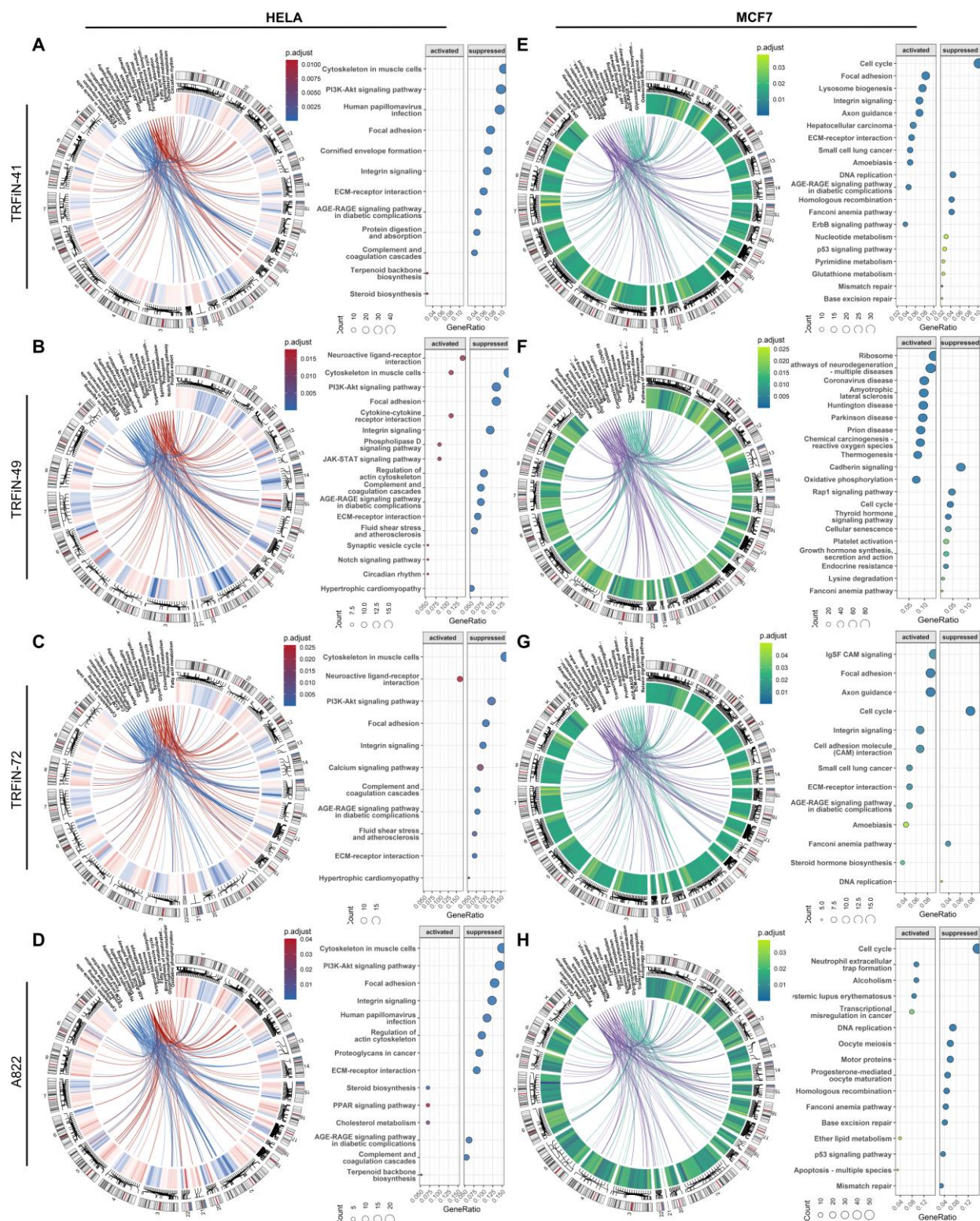


Figure 5.26. Chromosomal distribution of pathway associated genes and top 10 KEGG pathway enrichment after 168 h treatment with TRFiN compounds and A822. (A–D) Circos plots and bubble plots of TRFiN41, TRFiN49, TRFiN72, and A822 in HeLa cells, respectively. (E–H) Circos plots and bubble plots of TRFiN41, TRFiN49, TRFiN72, and A822 in MCF7 cells, respectively. Bubble plots show the top 10 activated and top 10 suppressed KEGG pathways for each comparison.

When the four selected biological themes are considered directly, the clearest difference appears between HeLa and MCF7. In HeLa cells, cell cycle and DNA replication were not among the dominant

enriched terms in the top pathway lists, even though earlier phenotypic assays had already shown replication stress and cell cycle redistribution. In this cell line, the stronger transcript level signature was loss of extracellular matrix, integrin, focal adhesion, and phosphatidylinositol 3-kinase/protein kinase B associated programmes, together with sterol metabolic changes under TRFiN41 and A822. In MCF7 cells, the pathway profile was more directly linked to reduced proliferative drive. TRFiN41, TRFiN72, and A822 all suppressed DNA replication and cell cycle pathways, and A822 additionally suppressed repair and p53 related pathways. The pathway enrichment analysis provided an overview of the major transcriptional changes after treatment, but this level of analysis mainly identifies clusters of enriched pathways and does not by itself fully resolve compound specific and cell line specific mechanisms. For this reason, the interpretation was not restricted to enrichment output alone. To obtain a more reliable view of the biological response to each compound in each cell line, the expression patterns of individual genes were examined in selected KEGG pathways rather than relying only on simple pathway enrichment. The main pathways chosen for this focused analysis were hsa04218 Cellular senescence, hsa04110 Cell cycle, hsa04210 Apoptosis, hsa03030 DNA replication, and hsa05200 Pathways in cancer. These pathways were selected because they cover the main processes that were expected to change after prolonged telomere directed treatment, including loss of proliferative capacity, replication disturbance, checkpoint control, cell fate transition, and cancer related signalling context.

Within these selected pathways, individual genes and their functional relationships were examined manually to identify treatment- and cell specific patterns that could not be adequately captured by pathway enrichment alone. This allowed the direction of change in key regulators, the balance between shared and distinct pathway nodes, and the overlap between pathways to be considered in a more structured way. In this framework, the interpretation was based not only on whether a pathway appeared enriched, but also on how central genes within that pathway behaved and how those changes connected with related pathways. This approach made it possible to more clearly define whether a given treatment was associated mainly with replication inhibition, cell cycle suppression, senescence associated signalling, an apoptotic response, or a mixed phenotype. On this basis, the proposed cell line specific and compound specific mechanisms of action for TRFiN41, TRFiN49, TRFiN72, and A822 are provided in the supplementary file.

5.19 Mechanism of action of TRFiN compounds based on pathway level transcriptomic analysis

TRFiN41, TRFiN49, and TRFiN72 were developed as derivatives of the parent compound A822 to interfere with the TRF1-TIN2 interaction within the shelterin complex. Since TRF1 and TIN2 are required for proper shelterin organisation at telomeres, inhibition of this interaction is expected to disturb telomere protection and telomere replication. To assess whether the transcriptional response supported this mechanism, the regulated genes were examined across four major KEGG pathways associated with the expected cellular outcome: DNA replication, cell cycle, cellular senescence, and apoptosis. The same pathway groups were compared in HeLa and MCF7 cells after treatment with A822 and the TRFiN derivatives (Figure 5.27).

The most consistent response was observed in the DNA replication pathway (hsa03030). Across both cell lines and all compounds, several genes encoding key replication proteins were downregulated. The most pronounced changes were observed for MCM5, POLA2, and POLD3, which were downregulated across all eight treatment conditions. These genes represent different parts of the replication machinery: MCM5 is part of the minichromosome maintenance helicase complex required for replication licensing and fork progression, POLA2 is part of DNA polymerase α /primase activity needed for replication initiation, and POLD3 is a subunit of DNA polymerase δ involved in lagging strand synthesis and repair associated DNA synthesis. Other replication genes, including LIG1, POLA1, POLE, MCM2, MCM3, MCM4, MCM6, PRIM1, and RFC3, were also frequently downregulated. This pattern indicates that A822 and its TRFiN derivatives do not affect only one replication factor, but suppress a broader replication programme involving origin licensing,

polymerase activity, clamp loading, and DNA ligation. This replication signature is consistent with the proposed telomere directed mechanism. TRF1 supports efficient replication through telomeric DNA, and disruption of the TRF1-TIN2 interaction can make telomeres difficult to replicate. In this setting, telomeric replication stress may act as the initiating event, followed by a wider suppression of the replication machinery. The recurrent upregulation of POLD4 in a subset of conditions may reflect a compensatory stress or repair associated DNA synthesis response rather than normal productive replication. The mixed regulation of RPA related genes between HeLa and MCF7 also suggests that both cell lines sense replication stress, but may process stalled replication intermediates differently.

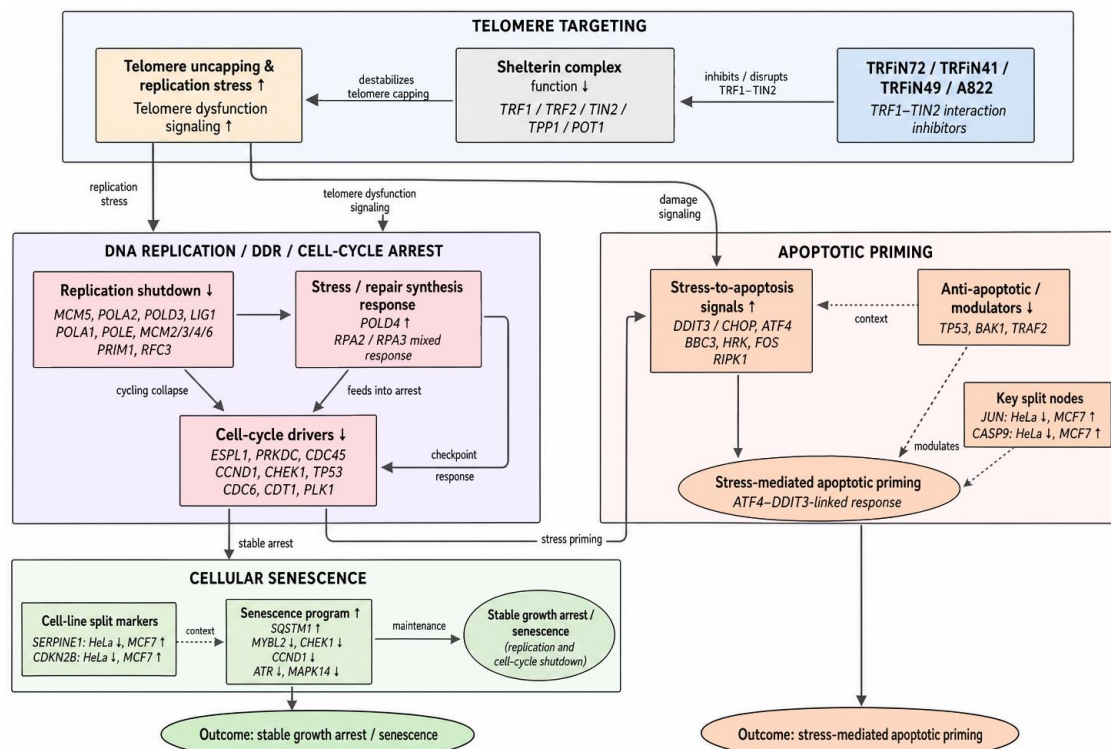


Figure 5.27. The schematic diagram of proposed common mechanism of action of TRFiN compounds. TRFiN72, TRFiN41, TRFiN49, and A822 inhibit the TRF1-TIN2 interaction, reducing shelterin function and inducing telomere uncapping, replication stress, and telomere dysfunction signalling. This leads to suppression of DNA replication and cell cycle genes, followed by growth arrest, senescence, and apoptotic priming. Solid arrows show proposed pathway flow, dashed arrows indicate context dependent regulation, and ↑/↓ indicate increased or decreased activity or gene expression.

The cell cycle pathway (hsa04110) supported the same interpretation. The genes ESPL1 and PRKDC were downregulated in all treatment conditions, while CDC45 was downregulated in most conditions. CDC45 is required for replication origin firing and CMG helicase function, linking the cell cycle response directly to the replication phenotype. Additional cell cycle drivers, including CCND1, CHEK1, MCM2, CDC6, CDT1, PLK1, BUB3, FBXO5, and TRIP13, were repeatedly decreased. These changes indicate suppression of G1/S transition, replication initiation, checkpoint supported S phase progression, and mitotic entry. Therefore, the transcriptional response suggests that the cells do not simply activate a transient checkpoint, but progressively lose the molecular programme required to maintain active proliferation. At the same time, several cytostatic regulators were increased. SMAD4 was upregulated in most conditions, and CDKN2D was also frequently increased. This indicates activation of growth inhibitory signalling in parallel with loss of cell cycle drivers. In MCF7 cells, CDK4 was often upregulated, but this was accompanied by downregulation of downstream replication and mitotic genes. This suggests that CDK4 upregulation does not represent productive proliferation, but may reflect a cell line specific compensatory or stalled G1 associated state. The combined downregulation of replication genes and mitotic regulators is more consistent with enforced cell cycle arrest.

The cellular senescence pathway (hsa04218) showed a conserved remodelling response after treatment with A822 and TRFiN compounds. The most consistent marker was SQSTM1/p62, which was upregulated in all treatment conditions. Since p62 is linked to autophagy, proteostasis, and stress adaptation, its induction suggests that treated cells undergo a stress remodelling process during growth arrest. This was accompanied by downregulation of MYBL2, CHEK1, and CCND1, all of which are associated with active cell cycle progression. Other genes such as ATR, MAPK14, MCU, PPP3CA, and TP53 were also frequently reduced. This expression pattern fits with a senescence like state in which proliferation genes are suppressed while stress adaptation pathways become more active. The senescence related response was not identical in HeLa and MCF7 cells. SERPINE1 and CDKN2B showed cell line dependent regulation, with stronger induction in MCF7 cells and reduced expression in HeLa cells. This suggests that the core arrest mechanism is shared between the two cell lines, but the senescence associated secretory and CDK inhibitory components differ according to cellular background. This difference is biologically reasonable because HeLa and MCF7 cells have distinct checkpoint, p53 related, and apoptotic wiring. Thus, the TRFiN compounds appear to induce a common anti-proliferative response, while the final senescence phenotype depends on the cell type.

The apoptosis (hsa04210) pathway showed a second major downstream branch of the response. The most consistent change was the upregulation of DDIT3/CHOP in all treatment conditions, together with frequent upregulation of ATF4. This points to activation of an integrated stress response or endoplasmic reticulum stress associated apoptotic programme. Several pro-apoptotic genes, including BBC3/PUMA, HRK, FOS, and RIPK1, were also frequently increased. These changes suggest that prolonged replication and telomere associated stress can prime cells for apoptosis. However, apoptosis execution was not uniform across the two cell lines. For example, JUN was consistently downregulated in HeLa but upregulated in MCF7, while CASP9 showed stronger induction in MCF7 than in HeLa. This indicates that the upstream stress apoptosis signal is common, but the downstream execution machinery is shaped by cell line specific signalling. The comparison between A822 and its derivatives suggests that the TRFiN compounds retain the parent compound's main biological direction, but converge more clearly on the expected shelterin linked phenotype. A822 and the TRFiN derivatives all produced suppression of DNA replication and cell cycle progression, together with induction of stress, senescence, and apoptosis related genes. This shared response supports the view that TRFiN41, TRFiN49, and TRFiN72 act through a mechanism related to the parent compound, but the TRFiN series represents a designed derivative group aimed more specifically at the TRF1-TIN2 interaction. The strongest common effect across all compounds was not immediate apoptosis alone, but replication shutdown followed by loss of proliferative capacity.

Based on these pathway level changes, the proposed mechanism is that TRFiN compounds inhibit the TRF1-TIN2 interaction and weaken shelterin function at telomeres. This causes telomere replication stress, which is reflected by suppression of replication licensing and polymerase genes such as MCM5, POLA2, and POLD3. Persistent replication stress then leads to downregulation of cell cycle drivers, including CDC45, CCND1, CHEK1, PLK1, and ESPL1, resulting in cell cycle arrest. Cells then move into two linked downstream outcomes: a senescence like growth arrest programme marked by SQSTM1/p62 induction and loss of proliferation associated genes, and a stress apoptosis programme marked by ATF4-DDIT3/CHOP activation and induction of pro-apoptotic factors such as BBC3/PUMA and HRK. The balance between senescence and apoptosis appears to differ between HeLa and MCF7 cells, but the initiating mechanism and the anti-replicative response are shared across the parent compound A822 and the TRFiN derivatives (Figure 5.27).

Chapter 6

Discussion

6.1 Targeting the TRF1-TIN2 interaction and relation among the findings

The parent compound A822, identified previously by our group, and its derivatives (TRFiN) were developed to target the TIN2 binding region within the TRFH domain of TRF1 protein. This region was selected because TIN2 binds directly to TRF1 and connects TRF1 with other shelterin proteins at telomeres. TIN2 was initially identified as a TRF1 binding protein involved in telomere length regulation, and later structural studies identified the pocket through which the TIN2 motif binds the TRF1-TRFH domain^{49,121}. The virtual screening shown in Figure 5.1 was therefore directed against this defined region. The SPR results in Figure 5.3 showed direct binding of A822 and several derivatives to purified TRF1, while the docking and molecular dynamics results in Figures 5.5 and 5.6 showed that the selected compounds remained within the proposed TIN2 binding region and formed repeated contacts with residues around the pocket. It is worth mentioning that TIN2 occupies a larger part of the TRFH domain compared to A822 and its derivatives as these ligands are smaller in size. Nevertheless, the interactions visible in *in silico* studies (docking and molecular dynamics) indicate that this binding is sufficient to interfere with TIN2 binding to TRF1. In MCF7 and HeLa cells, the compounds increased γ H2AX, increased the colocalisation of TIN2 with γ H2AX positive regions, disturbed replication fork progression and changed cell cycle distribution. Longer compound treatment produced telomere signal loss, telomere doublets and chromosome fusions, followed by senescence, mitochondrial damage and apoptosis. The compounds also reduced cell migration, colony formation, spheroid growth, vascular branching and *in ovo* microtumour growth.

The results obtained from the different methods followed the same biological sequence. SPR showed that the compounds bound TRF1, and the molecular simulations showed their interaction with the region used for compound design. Immunofluorescence experiments showed increased colocalisation of TIN2 and γ H2AX, which placed DNA damage signalling at TIN2 positive telomeric regions. Metaphase Telo-FISH showed structural telomere abnormalities, while the DNA fibre assay showed disturbed replication fork progression. The combination of SPR, molecular modelling, TIN2- γ H2AX colocalisation, Telo-FISH and DNA fibre results showed that TRF1 binding was associated with disturbance of the TRF1-TIN2 interaction and telomere replication stress. The cellular response developed with time. Changes in DNA replication, DNA damage signalling and cell cycle progression were detected before the largest increases in mitochondrial damage and apoptosis. Senescence associated staining and loss of colony forming ability became clearer after prolonged treatment. These results suggest that telomere and replication stress first reduced continued proliferation, after which the cells entered prolonged growth arrest or cell death according to the response of each cell line.

6.2 Cytotoxicity, selectivity and selection of lead compounds

The cytotoxicity results in Figure 5.2 divided the compound series into active and weak groups. TRFiN72 had the lowest median IC₅₀ among the synthesised derivatives at 6.12 μ M and was close to A822 at 6.35 μ M. TRFiN67b and TRFiN49 also remained within the low micromolar group, whereas TRFiN33, TRFiN45, TRFiN44 and TRFiN43 had considerably higher median values. Thus, only part of the derivative series retained the activity of the A822 scaffold. The compounds that were more active in the viability screening also produced stronger DNA damage and telomere related responses in the

later experiments. Testing in normal cells changed the ranking obtained from cancer cell activity alone. TRFiN72 showed the highest median selectivity index in the breast and colon panels, followed by TRFiN49. A822 was active against several cancer cell lines, but it was also highly active against human mammary epithelial cells and CCD 841 CoN cells. Its IC_{50} in CCD 841 CoN cells was lower than the values measured in the tested colon cancer lines. The comparison with MRC-5 cells gave a different result, and several active derivatives had selectivity indices below one. The selectivity of the compounds therefore depended on the normal cell model used for comparison. TRFiN72 gave the best result in the tissue matched breast and colon panels, although none of the compounds showed the same degree of selectivity in all normal cell models.

The lead compounds were selected by considering cytotoxicity, selectivity and TRF1 binding together. TRFiN72 was the most active derivative in the initial screening and showed the best selectivity in the breast and colon panels. TRFiN49 was the strongest derivative in the valid kinetic SPR ranking and remained highly active in the colony, spheroid and *in ovo* experiments. TRFiN41 produced strong cellular effects despite its weaker activity in the broad cytotoxicity screening and its weaker affinity for purified TRF1. A822 remained an important parent compound because it gave the lowest kinetic K_D , although its selectivity was poor in several matched normal cell comparisons. These differences explain why TRFiN41, TRFiN49 and TRFiN72 were selected for detailed experiments rather than choosing one compound from the IC_{50} results alone. The detailed cellular experiments were performed at the IC_{50} concentration of each compound. This allowed the responses to be examined at comparable levels of growth inhibition, although the molar concentration was not the same for every compound or cell line. The size of each response should therefore be considered together with the concentration used. The use of IC_{50} matched treatment was suitable for comparing the type and timing of the cellular changes produced by the selected compounds.

6.3 SPR and molecular simulation

The SPR sensorgrams in Figure 5.3 showed concentration dependent binding of A822 and several derivatives to immobilised TRF1. A822 gave the lowest fitted kinetic K_D among the small molecules at $0.58 \mu M$. TRFiN49 was the strongest derivative according to kinetic fitting, with a K_D of $6.83 \mu M$, followed by TRFiN67b and TRFiN38. TRFiN72 and TRFiN41 gave kinetic K_D values of 24.58 and $77.89 \mu M$, respectively. Physically meaningful association rate constants were not obtained for TRFiN43, TRFiN44 and TRFiN47, and their kinetic K_D values were therefore reported as not available. (The steady state values marked with double asterisks were mathematically obtained from curve fitting without a clear binding plateau and were excluded from the affinity ranking). The maximum SPR response did not follow the kinetic affinity order. TRFiN49 produced the highest response among the small molecules at approximately 202 RU, whereas A822 produced only approximately 24 RU despite having the lowest fitted kinetic K_D . TRFiN41 also produced a high response while showing the weakest valid kinetic affinity among the selected compounds. RU represents the amount of material associated with the sensor surface and is influenced by molecular mass, active concentration and accumulation at the surface. Therefore, a high RU value does not necessarily represent stronger affinity, and a low RU value does not necessarily represent weak binding. The sensorgram shape, response level and fitted binding constants were considered together when the SPR results were interpreted.

A822 showed a large difference between the kinetic and steady state analyses. Its kinetic K_D was $0.58 \mu M$, whereas its steady state K_D was $21.6 \mu M$. A822 is therefore described as the compound with the lowest fitted kinetic K_D rather than as the strongest binder according to every fitting method. TRFiN72 showed the closest agreement between the two analyses because its kinetic and steady state values remained within the same micromolar range. TRFiN49 had the lowest valid kinetic K_D among the derivatives, but its steady state value was mathematically obtained without a binding plateau and was not used for ranking. Based on the valid values, A822 had the lowest kinetic K_D , TRFiN49 was the strongest derivative by kinetic fitting, and TRFiN72 showed the closest agreement between kinetic

and steady state fitting. The TIN2 peptide produced the largest response at approximately 630 RU and gave a steady state K_D of 9.85 nM. Its kinetic K_D was 6.44 μ M, showing a large difference between the two fitting methods (Table 5.1). For the TIN2 peptide, the steady state value gave a clearer description of the interaction in this dataset because the peptide produced a strong concentration dependent response and represented the native TRF1 binding motif. The RU value of the peptide was not compared directly with the small molecule responses because the peptide and compounds differed in molecular mass and concentration range. The TIN2 peptide also confirmed that the immobilised TRF1 retained a functional TIN2 binding region. The amount of TRF1 immobilised on the sensor surface was high (10000 RU), which improved the detection of the small molecule responses. A dense protein surface can increase rebinding and mass transport effects, particularly when dissociation is slow or when kinetic and steady state fitting gives different values. This may have contributed to the separation between the kinetic and steady state K_D values of A822 and the TIN2 peptide. The direct binding result remained clear, but the affinity order was based mainly on physically meaningful kinetic fits, while the mathematically obtained steady state values were excluded.

The molecular dynamics results provided structural information that complemented the SPR analysis. The TRF1 protein backbone remained stable during the 1000 ns simulations, while the compounds differed in their movement within the binding region (Figure 5.5). TRFiN41 had the lowest mean ligand root mean square deviation, whereas A822 moved across a wider part of the pocket. TRFiN49 and TRFiN72 showed intermediate movement and maintained repeated contacts with residues around the TIN2 binding region. The MM/GBSA binding energy ranking placed TRFiN49 first, followed by A822, TRFiN72 and TRFiN41. This order agreed with the stronger SPR binding of TRFiN49 among the derivatives, although it did not reproduce the very low kinetic K_D obtained for A822. The difference between the simulation and SPR results was most evident for TRFiN41. Its movement within the modelled pocket was low, but its kinetic affinity was weaker than that of the other selected compounds. Molecular dynamics describes the behaviour of a ligand that has already been placed in a selected binding region, whereas SPR measures association from solution and dissociation from immobilised protein. Therefore, low movement within the simulated pocket can occur together with a weaker experimental K_D . The simulation results showed that the selected compounds could remain in the proposed region and form repeated residue contacts, while SPR provided the experimental measurement of direct TRF1 binding.

6.4 DNA damage, telomere abnormalities and replication stress

The γ H2AX results in Figure 5.4 clearly divided the active compounds from the weaker derivatives. After 168 h, A822, TRFiN41, TRFiN49 and TRFiN72 produced large γ H2AX positive populations in both cell lines, whereas TRFiN67b produced an intermediate response and most of the weak derivatives remained close to the control. HeLa cells showed the stronger late response, particularly after treatment with A822 and TRFiN72. The same active compounds also produced the main changes in the later mechanistic experiments, showing a relation between cytotoxic activity and sustained DNA damage signalling. The TIN2- γ H2AX colocalisation results in Figure 5.4C-E provided information on the association of DNA damage with TIN2 positive telomeric regions. Control cells showed little overlap between the TIN2 and γ H2AX antibody signals, whereas A822, TRFiN41, TRFiN49 and TRFiN72 increased the Pearson correlation coefficient after 72 h and produced a further increase after 168 h. TRFiN41 produced the highest early colocalisation in both cell lines. TIN2 is concentrated at shelterin containing telomeric regions, while γ H2AX marks chromatin undergoing a DNA damage response. The increased overlap between the antibody signals showed that γ H2AX positive DNA damage regions became more frequently associated with TIN2 positive telomeric regions during treatment. Earlier studies have shown that DNA damage proteins form telomere dysfunction induced foci when telomere protection is lost¹⁵². The Flow-FISH and metaphase Telo-FISH results in Figure 5.23 showed different levels of telomere change. Flow-FISH showed only a modest change in total telomere fluorescence, whereas metaphase analysis showed a much larger increase in structural abnormalities. Treated HeLa and MCF7 cells developed telomere signal loss,

telomere doublets and chromosome fusions. Telomere doublets are associated with fragile or incompletely replicated telomeres, while chromosome fusions indicate loss of normal chromosome end protection. These abnormalities were most pronounced after treatment with the main active compounds and were several fold higher than those observed in the control cells.

The limited change in bulk telomere fluorescence together with the marked increase in metaphase abnormalities is in agreement with disturbed telomere replication rather than rapid shortening of the complete telomere population. The treatment period lasted only several days, and extensive telomere shortening would not be expected during this period, particularly after cell proliferation had slowed. TRF1 is required for efficient replication of telomeric repeats and prevents telomere fragility caused by replication fork stalling. Recent research also showed that TRF1 supports fork reversal and restart at human telomeres. The telomere doublets and signal losses observed in the present study agree with these functions of TRF1^{192,93}. Previous studies showed that TRF1 inhibition reduced tumour growth by inducing telomeric DNA damage before substantial shortening of the overall telomere population occurred. Telomeric lesions can also maintain prolonged DNA damage signalling because repair is restricted within telomeric chromatin^{54,215}. These reports are in agreement with the strong γ H2AX induction, increased TIN2- γ H2AX colocalisation and structural telomere abnormalities observed in the present study despite only moderate changes in total telomere fluorescence.

The DNA fibre assay in Figure 5.9 showed disturbed progression of the two sequential nucleotide labelled tracts in both cell lines. TRFiN72 produced the largest reduction among the derivatives in HeLa cells, whereas TRFiN49 produced the largest reduction in MCF7 cells. These changes showed slower or less continuous replication fork progression after treatment. The assay measures replication across the genome, but the result agreed with the telomere doublets, the TIN2 associated γ H2AX signal and the known role of TRF1 in telomere replication. The DNA fibre results therefore placed replication stress among the earlier changes connecting TRF1 binding with the later chromosome end abnormalities. The TRF1/2-TIN2 colocalisation in Figure 5.23 remained within a similar range after treatment. Thus, the measured spatial overlap of TIN2 with the combined TRF1/2 signal was largely maintained while DNA damage and structural telomere abnormalities increased. A change in the function or organisation of the shelterin complex does not require complete removal or spatial separation of its proteins. The maintained TRF1/2-TIN2 colocalisation, together with the increased TIN2- γ H2AX overlap and metaphase telomere abnormalities, is consistent with functional disturbance at telomeres without a large loss of the measured shelterin associated signals.

6.5 Cell cycle changes, senescence and apoptosis

The cell cycle results in Figure 5.8 showed different responses in MCF7 and HeLa cells. MCF7 cells progressively accumulated in G0/G1 and lost much of the BrdU positive S phase population, with the clearest changes after 120 and 168 h. This response agreed with the reduced cell expansion, lower colony formation and later increase in senescence associated markers. HeLa cells showed an early increase in the BrdU positive population at 24 h, followed by changes at 72 h and later enrichment in G0/G1. The DNA fibre results showed that the early BrdU positive increase in HeLa cells did not represent normal rapid replication. Instead, the cells entered S phase but progressed through DNA synthesis under replication stress. The checkpoint proteins in Figure 5.24 followed the cell line dependent cell cycle responses. MCF7 cells showed the larger increase in the pCHK1/CHK1 ratio, whereas HeLa cells showed a very large increase in the pCHK2/CHK2 ratio; pCHK2 was not detected in MCF7 cells. The pATR/ATR and pATM/ATM ratios increased in both cell lines. This pattern is consistent with stronger CHK1 linked replication stress signalling in MCF7 cells and stronger CHK2 linked checkpoint signalling in HeLa cells. Cyclin E1 decreased in both cell lines, whereas cyclin D1 increased strongly in MCF7 and decreased under most treatments in HeLa. Although the checkpoint and cyclin responses differed between the two cell lines, both responses resulted in reduced cell cycle progression.

Senescence associated changes appeared after the earlier replication and DNA damage responses. Senescence associated fluorescence remained low at 24 h and increased from 72 h onward. At 168 h, X-gal staining showed increased senescence associated β galactosidase activity together with enlarged cell morphology. Lamin B1 decreased and p27 increased in both cell lines. Loss of Lamin B1 is a recognised marker of cellular senescence, and previous studies have linked telomere initiated senescence with a sustained DNA damage checkpoint^{27,216}. The marked reduction in colony formation further showed that many surviving cells had lost their long term ability to proliferate. The protein changes associated with senescence were not identical in MCF7 and HeLa cells. p21 did not increase uniformly and was therefore not the main common mediator of the response. In contrast, p27 increased in both cell lines, while p16 was detected more clearly in HeLa and was not detected in MCF7. The senescence response was therefore identified from the increase in cell size and β galactosidase staining together with Lamin B1 loss, p27 induction, G0/G1 accumulation and reduced colony formation. The different p21 and p16 responses reflect the different cellular backgrounds of MCF7 and HeLa cells.

Mitochondrial damage became clearer during prolonged treatment. The JC-1 results in Figure 5.11 showed loss of mitochondrial membrane potential in both cell lines. MitoTracker and TOMM20 imaging showed fragmented and clustered mitochondrial networks, while transmission electron microscopy in Figure 5.12 showed swollen and irregular mitochondria with damaged internal organisation. AIF staining became less continuous with TOMM20 and accumulated towards the perinuclear region. The flow cytometry, fluorescence imaging and ultrastructural results all showed that mitochondrial damage developed during the later cellular response. Previous studies using genetic models showed that telomere dysfunction activated p53, which suppressed PGC-1 regulators and reduced mitochondrial function²¹⁷. The experimental model in that report differed from the cancer cell treatments used here, but it provides a biological link between persistent telomere damage and mitochondrial decline. In the present study, replication disturbance and γ H2AX induction appeared before the clearest mitochondrial changes. This time course suggests that mitochondrial damage developed after sustained DNA and telomere stress rather than being the first cellular response to treatment. Apoptosis increased after prolonged treatment, but the pathway differed between the two cell lines. HeLa cells showed late annexin V positivity, increased caspase-3/7 activity and PARP degradation, indicating caspase dependent apoptosis. MCF7 cells showed annexin positive populations, loss of mitochondrial membrane potential and changes in AIF without the same PARP response. MCF7 cells lack functional caspase-3, but apoptosis can proceed through caspase-7 and caspase independent mitochondrial pathways in this cell line²¹⁸. Bcl-2 decreased and PUMA increased in both cell lines, while BAX increased more strongly in MCF7 and showed a variable response in HeLa. These results show that prolonged treatment induced apoptosis through cell line dependent execution pathways.

6.6 Migration, long term growth and *in ovo* activity

Live cell tracking and wound healing analysis in Figures 5.7 and 5.16 showed reduced cell movement and slower repopulation of the wound area after treatment. TRFiN49 and 4-hydroxytamoxifen produced the strongest restriction of movement in MCF7 cells, whereas TRFiN41 produced a clear response in HeLa cells. RhoA and N-cadherin decreased in the Western blot analysis, in agreement with the changes in cell motility and adhesion. Since wound closure depends on movement, proliferation and survival, the slower closure reflects the combined reduction of these processes rather than movement alone. The colony and spheroid experiments showed that the compound activity continued beyond the initial viability assay. TRFiN49 strongly reduced colony formation in both cell lines and remained active in three dimensional spheroids. TRFiN41 produced a particularly strong response in MCF7 spheroids, while TRFiN72 became more active at higher concentrations in several long term experiments. Three dimensional cultures differ from monolayers in cell contact, oxygen and nutrient distribution and compound penetration. Previous studies reported lower responses to several established anticancer drugs in spheroids than in matched

monolayers²¹⁹. The reduction of spheroid growth observed here showed that the TRFiN compounds retained their activity under three dimensional growth conditions. Acridine orange staining in Figure 5.19 showed increased acidic vesicular organelles, particularly in MCF7 cells treated with TRFiN72. This result showed an increase in acidic lysosomal or autophagy associated compartments during cellular stress. The stronger response in MCF7 than in HeLa agreed with the other cell line differences observed in mitochondrial and apoptotic signalling. SQSTM1 also increased in the transcriptomic analysis. The present results are therefore described as accumulation of acidic vesicular organelles and altered vesicle associated stress rather than as a complete measurement of autophagic flux.

The chorioallantoic membrane (CAM) assay in Figure 5.20 showed little change after 6 h, followed by a clearer reduction in vascular branching after 24 and 48 h. TRFiN49 produced the strongest response at 48 h, followed by A822, TRFiN41 and TRFiN72. The later reduction in vascular branching agreed with the gradual development of the cellular stress response observed *in vitro*. Vascular branching depends on endothelial cell movement, proliferation and survival, and the assay measured the combined effect of the compounds on these processes. The results showed that the lead compounds reduced vascular development under the tested conditions. Treatment also reduced the weight of HeLa and MCF7 microtumours in the *in ovo* model shown in Figure 5.21. This result agreed with the colony and spheroid experiments and showed that the compounds remained active in a more complex biological system. Previous inhibition of TRF1 also reduced tumour growth through telomeric DNA damage in mouse cancer models⁵⁴. Although the models and treatment schedules were different, both studies showed reduced tumour growth after disturbance of TRF1 related telomere protection. Histological examination in Figure 5.22 did not show visible structural injury in the analysed embryonic organs at the tested dose. The active treatment therefore did not produce clear organ damage during the short observation period. This finding should be considered together with the normal cell IC₅₀ results, which showed that selectivity was not uniform in all cell models. Wider dose testing and mammalian exposure studies are required to determine the safety range, while the *in ovo* histology provides support for further evaluation of the lead compounds.

6.7 Protein expression and transcriptomic changes and comparison of the lead compounds

The protein expression changes in Figure 5.24 were in agreement with the main cellular results. Increased γ H2AX together with increased pATM/ATM and pATR/ATR ratios accompanied DNA damage and replication stress. The larger pCHK1/CHK1 response in MCF7 and the strong pCHK2/CHK2 response in HeLa agreed with the different cell cycle patterns. These values represent phosphorylated to total protein ratios and should not be described as fold changes in the phosphorylated bands alone. Lower Lamin B1 and higher p27 accompanied senescence associated arrest, while lower Bcl-2, higher PUMA and PARP degradation in HeLa accompanied delayed apoptosis. RhoA and N-cadherin decreased together with the reduction in wound closure. The combined TRF1/2 signal remained relatively close to the control, indicating that the cellular response occurred without a large reduction in this measured signal. Because the antibody signal did not distinguish TRF1 from TRF2, this result should be interpreted as supporting a functional change in shelterin rather than as direct evidence for unchanged TRF1 abundance. One clear difference between the protein and transcript data concerned Cyclin D1 in MCF7 cells: the Western blot showed a large increase in Cyclin D1 protein, whereas the transcriptomic analysis identified CCND1 among the repeatedly decreased cell cycle genes. These measurements were therefore not concordant for Cyclin D1 and should be interpreted at their respective protein and mRNA levels. The transcriptome was analysed after 168 h, when growth arrest, senescence and apoptosis were already present. The expression data therefore described the late cellular response to treatment. HeLa cells showed the largest number of differentially expressed genes after TRFiN41 treatment, whereas MCF7 cells showed the largest response after TRFiN49 treatment. In HeLa cells, reduced extracellular matrix, focal adhesion and cytoskeletal pathways agreed with the lower cell movement and wound

repopulation. In MCF7 cells, the stronger reduction of DNA replication, cell cycle and DNA repair pathways agreed with G0/G1 accumulation and loss of the BrdU positive population.

Several replication associated genes changed in the same direction across the compound and cell line comparisons. MCM5, POLA2 and POLD3 decreased in all eight conditions, while LIG1, POLA1, POLE, other MCM genes, PRIM1, RFC3 and CDC45 decreased after several treatments. ESPL1 and PRKDC also decreased across most conditions. These changes agreed with the DNA fibre and cell cycle results and showed reduced expression of genes required for DNA synthesis and cell division after prolonged treatment. DDIT3 increased in every condition and ATF4 increased in several conditions, showing a common cellular stress response. The transcriptomic analysis results therefore extended the protein and cell based findings to the pathway level. A822 and the three lead derivatives showed different strengths across the experiments. A822 gave the lowest fitted kinetic K_D and remained an active parent scaffold, but its steady state K_D was higher and its selectivity was weak in the tissue matched breast and colon comparisons. TRFiN49 gave the lowest valid kinetic K_D among the derivatives and the most favourable MM/GBSA result. It also produced strong inhibition of colony formation and spheroid growth, the greatest reduction in vascular branching and the largest transcriptomic response in MCF7 cells. When TRF1 binding and long term cellular activity were considered together, TRFiN49 showed the strongest result among the derivatives.

TRFiN72 had the lowest median IC_{50} among the derivatives and showed the best selectivity in the breast and colon panels. Its kinetic and steady state SPR values also remained within the same micromolar range. TRFiN72 produced strong γ H2AX induction, replication fork disturbance, telomere abnormalities and reduction of *in ovo* tumour growth. TRFiN41 had the weakest valid kinetic affinity among the main compounds but produced strong DNA damage, senescence associated changes and marked cellular responses in both HeLa and MCF7 cells. This difference may be related to compound uptake, intracellular retention or other intracellular properties. The three derivatives were therefore selected for different reasons: TRFiN49 for its combined binding and long term activity, TRFiN72 for cytotoxic potency and selectivity, and TRFiN41 for its strong cellular response.

6.8 Limitations and scope for further research

This study also has some limitations that should be considered for further studies. One major limitation was the lack of a specific and reliable antibody that could detect TRF1 exclusively under the required experimental conditions. All available antibodies that could be obtained were tested, but none of them gave a satisfactory and consistent result. Because of this limitation, direct cellular confirmation of TRF1 behaviour after compound treatment could not be completed at the same level as the downstream functional assays. Another important limitation is related to the nature of the target itself. The TRF1-TIN2 axis is a protein-protein interaction and inhibition of this type of target may not produce a rapid cellular response like inhibition of an enzyme does. There is also no single gold standard experiment that can prove TRF1-TIN2 inhibition inside cells without limitation. For this reason, the mechanism had to be examined through several downstream effects of telomere dysfunction, including telomere associated DNA damage, replication stress, telomere aberrations, cell cycle arrest, senescence and apoptosis. The present results also clarify an important point in telomere biology. Telomere damage does not always require rapid telomere shortening before cellular effects appear. In the present model, even if the TRF1-TIN2 interaction is disturbed, shelterin proteins may still remain associated with telomeric DNA and may protect many chromosome ends. However, a part of the telomere population may become more prone to double strand break formation during replication, and these damaged telomeres can play a key role in activating the downstream response. Another limitation is that TRF1-TIN2 interaction is also present in normal cells. Therefore, compounds directed against this interaction may also affect normal proliferating cells. Because of this, the compounds described in this study should be considered first as experimental inhibitors of the TRF1-TIN2 interaction. Their future therapeutic use would require a more selective delivery system. Site directed delivery approaches such as nanocomplex based delivery, liposome mediated delivery or

other tumour targeted carrier systems may help to improve cancer cell selectivity and reduce unwanted effects in normal tissues. The activity of these compounds should also be examined in combination with other anticancer agents. This is important because most cancer treatments are not given as single drugs, but as part of combination therapy. Further studies should therefore evaluate whether TRFiN compounds can act together with DNA damage response inhibitors, replication stress inducing agents or standard chemotherapeutic drugs, and whether such combinations can improve anticancer activity while maintaining an acceptable toxicity profile.

Chapter 7

Conclusion

This thesis describes the development and biological evaluation of novel small molecules targeting the interaction between TRF1 and TIN2 within the shelterin complex. The parent compound A822 was previously identified by our group, and a new series of A822 derivatives, named TRFiN compounds, was prepared to improve its biological activity and anticancer properties. To the best of our knowledge, this is the first study describing novel small molecules developed against the TRF1-TIN2 protein-protein interaction and the first comprehensive account of the cellular mechanism and consequences of disturbing this interaction in cancer cells. The results show that selected TRFiN compounds bind TRF1 and are predicted to occupy the TIN2 binding region and affect the functional organisation of shelterin, leading to telomere replication stress, telomere associated DNA damage signalling and loss of sustained cancer cell proliferation. Disturbance of the TRF1-TIN2 interaction does not require the complete removal of either protein from telomeres. TRF1 can remain attached to double stranded telomeric DNA through its MYB DNA binding domain, while TIN2 can remain associated with the shelterin complex through its interaction with TRF2 and other shelterin proteins. Therefore, disruption of the TRF1-TIN2 interface can change the organisation and function of shelterin without producing complete spatial separation of TRF1 and TIN2. This is consistent with the maintained TRF1/2-TIN2 colocalisation measured after treatment, despite increased DNA damage signalling and telomere abnormalities. The compounds appear to disturb the functional arrangement of shelterin rather than removing the complete complex from chromosome ends.

During the earlier stages of treatment, the remaining shelterin contacts may continue to provide partial protection to many telomeres. However, disturbance of the TRF1-TIN2 interface can make some telomeric regions more difficult to replicate and more vulnerable to DNA damage. This is particularly important and possible when the whole shelterin complex is undergoing rearrangement during cell cycle and TRF1 is more exposed to interaction with external molecules, including our ligands. This was reflected by slower replication fork progression, increased γ H2AX and greater colocalisation of TIN2 with γ H2AX positive regions. Western blotting further supported this cell line dependent checkpoint response: pCHK1/CHK1 increased more strongly in MCF7 cells, pCHK2/CHK2 increased very strongly in HeLa cells and was not detected in MCF7 cells, while pATM/ATM and pATR/ATR increased in both cell lines. Lamin B1 decreased and p27 increased in both cell lines, while Bcl-2 decreased and PUMA increased; BAX increased more strongly in MCF7 cells and PARP degradation was observed in HeLa cells. At this stage, the remaining shelterin organisation may still be sufficient to restrict extensive chromosome end joining and recombination, even though local telomere damage and replication stress have already developed. The early response therefore represents partial loss of telomere function rather than complete removal of chromosome end protection. With continued treatment, telomere associated damage became more extensive and was accompanied by telomere signal loss, telomere doublets, chromosome fusions and other chromosomal abnormalities. Persistent or incorrectly repaired lesions at chromosome ends may allow inappropriate DNA repair activity and promote the joining or rearrangement of damaged chromosomes. These changes were followed by cell cycle arrest, loss of colony forming ability, mitochondrial damage, senescence associated changes and apoptosis. The response developed gradually, with replication disturbance and DNA damage signalling appearing before the strongest mitochondrial and apoptotic

changes. This indicates that mitochondrial injury and cell death were later consequences of prolonged telomere and replication stress rather than the first effects of compound treatment.

The treatment produced only moderate changes in total telomere fluorescence, although structural telomere abnormalities increased markedly. This shows that the compounds did not act mainly by rapidly shortening the complete telomere population. Instead, they disturbed the protection, replication and structural stability of chromosome ends. Telomeres could therefore retain much of their repeat length while becoming functionally damaged. The remaining shelterin contacts may continue to protect part of the telomere population, while telomeres with greater disturbance accumulate replication defects, DNA damage signalling and incorrect chromosome end repair. The resulting chromosomal instability can restrict cell proliferation without requiring extensive telomere shortening. The cellular pathway following telomere damage differed between MCF7 and HeLa cells. MCF7 cells showed stronger G0/G1 accumulation and a response associated with senescence and mitochondrial dysfunction, whereas HeLa cells showed stronger late caspase activation, PARP degradation and apoptosis. These differences reflect the distinct genetic and signalling backgrounds of the two cell lines. However, the final biological outcome was similar. Continued proliferation was reduced, colony and spheroid growth were inhibited, cell migration decreased and part of the treated population entered prolonged growth arrest while another part underwent cell death. The findings of this thesis support a mechanism in which A822 derived TRFiN compounds disturb the TRF1-TIN2 interaction and weaken the functional organisation of shelterin without requiring a major loss of the measured TRF1/2-TIN2 association. This disturbance increases telomere replication stress and DNA damage signalling, followed by structural telomere abnormalities, chromosomal instability and loss of proliferative capacity. Depending on the cellular background and the extent of accumulated damage, the treated cells enter senescence associated arrest or apoptosis. The main action of these compounds is therefore related to functional telomere damage rather than rapid telomere shortening. This identifies the TRF1-TIN2 interaction as a suitable target for the development of telomere directed anticancer compounds.

Future research should examine the toxicity and selectivity of the lead compounds in human derived models, including normal and tumour organoids that more closely represent human tissue organisation. Detailed dose response studies are required to define the concentration range that produces telomere dysfunction while limiting damage to normal cells. Possible synergistic activity of the TRFiN compounds with established anticancer drugs, DNA damage response inhibitors and other telomere directed treatments should also be examined. A faster and standardised screening method should be developed by combining measurements of TRF1 binding, telomere associated DNA damage, replication stress and cell fate changes within a shorter experimental period. Such a method would allow larger numbers of derivatives to be compared and would support the development of more active, selective and safer TRF1-TIN2 targeting drug candidates.

Bibliography

1. Bray F, Laversanne M, Sung H, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2024; 74: 229-63
2. Hanahan D. Hallmarks of cancer: new dimensions. *Cancer Discov* 2022; 12: 31-46
3. van Emden TS, Forn M, Forné I, et al. Shelterin and subtelomeric DNA sequences control nucleosome maintenance and genome stability. *EMBO Rep* 2019; 20: e47181
4. Luo Q, Smith DP. Global cancer burden: progress, projections, and challenges. *Lancet* 2025; 406: 1536-7
5. Housman G, Byler S, Heerboth S, et al. Drug resistance in cancer: an overview. *Cancers (Basel)* 2014; 6: 1769-92
6. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013; 13: 714-26
7. Gupta GP, Massagué J. Cancer metastasis: building a framework. *Cell* 2006; 127: 679-95
8. Yang K, Yang C, Xiong K, Hao J, Ye L. Adaptive resistance in cancer immunotherapy. *Cell Mol Immunol* 2026; 23: 743-72
9. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011; 331: 1559-64
10. Oh BY, Shin H-T, Yun JW, et al. Intratumor heterogeneity inferred from targeted deep sequencing as a prognostic indicator. *Sci Rep* 2019; 9: 4542
11. Zhang J, Li H, Ru S, et al. Tumor heterogeneity: development, mechanisms, and therapeutic implications. *Sig Transduct Target Ther* 2026; 11: 271
12. Sanglier C, Cayrefourcq L, Alix-Panabières C. Reconstructing the metastatic journey: functional circulating tumor cells and disseminated tumor cells based models for translational oncology. *J Exp Clin Cancer Res* 2026; 45: 46
13. Kreso A, O'Brien CA, van Galen P, et al. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. *Science* 2013; 339: 543-8
14. Iskandar M, Xiao Barbero M, Jaber M, et al. A review of telomere attrition in cancer and aging: current molecular insights and future therapeutic approaches. *Cancers* 2025; 17: 257
15. de Bardet JC, Cardentey CR, González BL, et al. Cell immortalization: in vivo molecular bases and in vitro techniques for obtention. *BioTech* 2023; 12: 14
16. Nassour J, Schmidt TT, Karlseder J. Telomeres and cancer: resolving the paradox. *Annu Rev Cancer Biol* 2021; 5: 59-77
17. Carneiro T, Khair L, Reis CC, et al. Telomeres avoid end detection by severing the checkpoint signal transduction pathway. *Nature* 2010; 467: 228-32
18. Rembiałkowska N, Sędzik M, Kisielewska M, et al. Telomere maintenance and DNA repair: a bidirectional relationship in cancer biology and therapy. *Cancers* 2025; 17: 2284
19. Palm W, Lange T de. How shelterin protects mammalian telomeres. *Annu Rev Genet* 2008; 42: 301-34
20. de Lange T. How telomeres solve the end-protection problem. *Science* 2009; 326: 948-52
21. Lim CJ, Cech TR. Shaping human telomeres: from shelterin and CST complexes to telomeric chromatin organization. *Nat Rev Mol Cell Biol* 2021; 22: 283-98

22. Griffith JD, Comeau L, Rosenfield S, et al. Mammalian telomeres end in a large duplex loop. *Cell* 1999; 97: 503-14
23. de Lange T. Shelterin-mediated telomere protection. *Annu Rev Genet* 2018; 52: 223-47
24. Ruis P, Boulton SJ. The end protection problem-an unexpected twist in the tail. *Genes Dev* 2021; 35: 1-21
25. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol* 1973; 41: 181-90
26. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990; 345: 458-60
27. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426: 194-8
28. Di Micco R, Krizhanovsky V, Baker D, d'Adda di Fagagna F. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat Rev Mol Cell Biol* 2021; 22: 75-95
29. Hayashi MT, Cesare AJ, Rivera T, Karlseder J. Cell death during crisis is mediated by mitotic telomere deprotection. *Nature* 2015; 522: 492-6
30. Akincilar SC, Unal B, Tergaonkar V. Reactivation of telomerase in cancer. *Cell Mol Life Sci* 2016; 73: 1659-70
31. Sharma S, Chowdhury S. Emerging mechanisms of telomerase reactivation in cancer. *Trends Cancer* 2022; 8: 632-41
32. Keam SJ. Imetelstat: first approval. *Drugs* 2024; 84: 1149-55
33. Patel H, Yohannes S, Brunaugh E. Imetelstat: a first-in-class telomerase inhibitor for the treatment of patients with lower-risk myelodysplastic syndromes and anemia. *J Adv Pract Oncol* 2025; 1-7
34. Bruedigam C, Porter AH, Song A, et al. Imetelstat-mediated alterations in fatty acid metabolism to induce ferroptosis as a therapeutic strategy for acute myeloid leukemia. *Nat Cancer* 2024; 5: 47-65
35. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. *Nat Rev Genet* 2010; 11: 319-30
36. Zhang J-M, Genois M-M, Ouyang J, Lan L, Zou L. Alternative lengthening of telomeres is a self-perpetuating process in ALT-associated PML bodies. *Mol Cell* 2021; 81: 1027-1042.e4
37. Bilsland AE, Cairney CJ, Keith WN. Targeting the telomere and shelterin complex for cancer therapy: current views and future perspectives. *J Cell Mol Med* 2011; 15: 179-86
38. Bruedigam C, Lane SW. Telomerase in hematologic malignancies. *Curr Opin Hematol* 2016; 23: 346-53
39. Fairall L, Chapman L, Moss H, de Lange T, Rhodes D. Structure of the TRFH dimerization domain of the human telomeric proteins TRF1 and TRF2. *Mol Cell* 2001; 8: 351-61
40. Hu C, Rai R, Huang C, et al. Structural and functional analyses of the mammalian TIN2-TPP1-TRF2 telomeric complex. *Cell Res* 2017; 27: 1485-502
41. Denchi EL, de Lange T. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature* 2007; 448: 1068-71
42. Sfeir A, de Lange T. Removal of shelterin reveals the telomere end-protection problem. *Science* 2012; 336: 593-7
43. Wieczór M, Tobiszewski A, Wityk P, Tomiczek B, Czub J. Molecular recognition in complexes of TRF proteins with telomeric DNA. *PLoS One* 2014; 9: e89460
44. Chen Y. The structural biology of the shelterin complex. *Biol Chem* 2019; 400: 457-66

45. Salvati E, Leonetti C, Rizzo A, et al. Telomere damage induced by the G-quadruplex ligand RHPS4 has an antitumor effect. *J Clin Invest* 2007; 117: 3236-47
46. Gomez D, O'Donohue M-F, Wenner T, et al. The G-quadruplex ligand telomestatin inhibits POT1 binding to telomeric sequences in vitro and induces GFP-POT1 dissociation from telomeres in human cells. *Cancer Res* 2006; 66: 6908-12
47. Yu S, Wei S, Savani M, et al. A modified nucleoside 6-thio-2'-deoxyguanosine exhibits antitumor activity in gliomas. *Clin Cancer Res* 2021; 27: 6800-14
48. Mender I, Gryaznov S, Dikmen ZG, Wright WE, Shay JW. Induction of telomere dysfunction mediated by the telomerase substrate precursor 6-thio-2'-deoxyguanosine. *Cancer Discov* 2015; 5: 82-95
49. Chen Y, Yang Y, van Overbeek M, et al. A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins. *Science* 2008; 319: 1092-6
50. Lim CJ, Zaug AJ, Kim HJ, Cech TR. Reconstitution of human shelterin complexes reveals unexpected stoichiometry and dual pathways to enhance telomerase processivity. *Nat Commun* 2017; 8: 1075
51. Janovič T, Stojaspal M, Veverka P, Horáková D, Hofr C. Human telomere repeat binding factor TRF1 replaces TRF2 bound to shelterin core hub TIN2 when TPP1 is absent. *J Mol Biol* 2019; 431: 3289-301
52. Zinder JC, Olinares PDB, Svetlov V, et al. Shelterin is a dimeric complex with extensive structural heterogeneity. *Proc Natl Acad Sci U S A* 2022; 119: e2201662119
53. Janovič T, Perez GI, Schmidt JC. TRF1 and TRF2 form distinct shelterin subcomplexes at telomeres. *bioRxiv* 2024; 2024.12.23.630076
54. García-Beccaria M, Martínez P, Méndez-Pertuz M, et al. Therapeutic inhibition of TRF1 impairs the growth of p53-deficient K-RasG12V-induced lung cancer by induction of telomeric DNA damage. *EMBO Mol Med* 2015; 7: 930-49
55. Bejarano L, Louzame J, Montero JJ, Megías D, Flores JM, Blasco MA. Safety of whole-body abrogation of the TRF1 shelterin protein in wild-type and cancer-prone mouse models. *iScience* 2019; 19: 572-85
56. Casale G, Liu M, Le Bihan Y-V, et al. Discovery of first-in-class inhibitors of the TRF1:TIN2 protein:protein interaction by fragment screening. *Sci Rep* 2025; 15: 40922
57. Brankiewicz W, Kalathiya U, Padariya M, et al. Modified peptide molecules as potential modulators of shelterin protein functions; TRF1. *Chem Eur J* 2023; 29: e202300970
58. Lennox AL, Huang F, Behrs MK, et al. Imetelstat, a novel, first-in-class telomerase inhibitor: mechanism of action, clinical, and translational science. *Clin Transl Sci* 2024; 17: e70076
59. Oh B-K, Kim Y-J, Park C, Park YN. Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. *Am J Pathol* 2005; 166: 73-80
60. Bagiński M, Skowron P, Żebrowska J, et al. Compound which inhibits telomere-binding protein, and telomere-binding protein inhibitor containing same [Internet]. 2023 [cited 2026 June 30]. Available from: <https://patents.google.com/patent/WO2023182897A1/fr>
61. de Lange T. How shelterin orchestrates the replication and protection of telomeres. *Cold Spring Harb Perspect Biol* 2025; a041685
62. Lei M, Podell ER, Cech TR. Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol* 2004; 11: 1223-9
63. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005; 19: 2100-10

64. Rai R, Chen Y, Lei M, Chang S. TRF2-RAP1 is required to protect telomeres from engaging in homologous recombination-mediated deletions and fusions. *Nat Commun* 2016; 7: 10881
65. Arat NÖ, Griffith JD. Human Rap1 interacts directly with telomeric DNA and regulates TRF2 localization at the telomere. *J Biol Chem* 2012; 287: 41583-94
66. Bank RPD. RCSB PDB - 3BQO: Crystal Structure of TRF1 TRFH domain and TIN2 peptide complex [Internet]. [cited 2025 Dec 30]. Available from: https://www.rcsb.org/structure/3BQO?utm_source=chatgpt.com
67. Pan H, Kaur P, Barnes R, et al. Structure, dynamics, and regulation of TRF1-TIN2-mediated trans- and cis-interactions on telomeric DNA. *J Biol Chem* 2021; 297: 101080
68. Kim H, Lee O-H, Xin H, et al. TRF2 functions as a protein hub and regulates telomere maintenance by recognizing specific peptide motifs. *Nat Struct Mol Biol* 2009; 16: 372-9
69. O'Connor MS, Safari A, Xin H, Liu D, Songyang Z. A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. *Proc Natl Acad Sci U S A* 2006; 103: 11874-9
70. Pike AM, Strong MA, Ouyang JPT, Greider CW. TIN2 functions with TPP1/POT1 to stimulate telomerase processivity. *Mol Cell Biol* 2019; 39: e00593-18
71. Bejarano L, Schuhmacher AJ, Méndez M, et al. Inhibition of TRF1 telomere protein impairs tumor initiation and progression in glioblastoma mouse models and patient-derived xenografts. *Cancer Cell* 2017; 32: 590-607.e4
72. Bank RPD. RCSB PDB - 9HHK: Crystal structure of human TRF1 TRFH domain in complex with compound 2 [Internet]. [cited 2025 Dec 30]. Available from: https://www.rcsb.org/structure/9HHK?utm_source=chatgpt.com
73. Janovič T, Perez GI, Boelting G, Schmidt JC. TRF1 and TRF2 form distinct shelterin subcomplexes at telomeres. *Cell Rep* 2025; 44: 116178
74. Erdel F, Kratz K, Willcox S, Griffith JD, Greene EC, de Lange T. Telomere recognition and assembly mechanism of mammalian shelterin. *Cell Rep* 2017; 18: 41-53
75. Hu H, Yan HL, Nguyen THD. Structural biology of shelterin and telomeric chromatin: the pieces and an unfinished puzzle. *Biochem Soc Trans* 2024; 52: 1551-64
76. Veverka P, Janovič T, Hofr C. Quantitative biology of human shelterin and telomerase: searching for the weakest point. *Int J Mol Sci* 2019; 20: 3186
77. Doksani Y, de Lange T. Telomere-internal double-strand breaks are repaired by homologous recombination and PARP1/Lig3-dependent end-joining. *Cell Rep* 2016; 17: 1646-56
78. Doksani Y, Wu JY, de Lange T, Zhuang X. Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation. *Cell* 2013; 155: 345-56
79. Benarroch-Popivker D, Pisano S, Mendez-Bermudez A, et al. TRF2-mediated control of telomere DNA topology as a mechanism for chromosome-end protection. *Mol Cell* 2016; 61: 274-86
80. Celli GB, Denchi EL, de Lange T. Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. *Nat Cell Biol* 2006; 8: 885-90
81. Bombarde O, Boby C, Gomez D, et al. TRF2/RAP1 and DNA-PK mediate a double protection against joining at telomeric ends. *EMBO J* 2010; 29: 1573-84
82. Bae NS, Baumann P. A RAP1/TRF2 complex inhibits nonhomologous end-joining at human telomeric DNA ends. *Mol Cell* 2007; 26: 323-34
83. Tesmer VM, Brenner KA, Nandakumar J. Human POT1 protects the telomeric ds-ss DNA junction by capping the 5' end of the chromosome. *Science* 2023; 381: 771-8
84. Pinzaru AM, Hom RA, Beal A, et al. Telomere replication stress induced by POT1 inactivation accelerates tumorigenesis. *Cell Rep* 2016; 15: 2170-84
85. Kibe T, Zimmermann M, de Lange T. TPP1 blocks an ATR-mediated resection mechanism at telomeres. *Mol Cell* 2016; 61: 236-46

86. Wu P, van Overbeek M, Rooney S, de Lange T. Apollo contributes to G overhang maintenance and protects leading-end telomeres. *Mol Cell* 2010; 39: 606-17
87. Lam YC, Akhter S, Gu P, et al. SNMIB/Apollo protects leading-strand telomeres against NHEJ-mediated repair. *EMBO J* 2010; 29: 2230-41
88. Konishi A, Lange T de. Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2. *Genes Dev* 2008; 22: 1221-30
89. Nishikawa T, Okamura H, Nagadoi A, König P, Rhodes D, Nishimura Y. Solution structure of a telomeric DNA complex of human TRF1. *Structure* 2001; 9: 1237-51
90. König P, Fairall L, Rhodes D. Sequence-specific DNA recognition by the Myb-like domain of the human telomere binding protein TRF1: a model for the protein-DNA complex. *Nucleic Acids Res* 1998; 26: 1731-40
91. Boskovic J, Martinez-Gago J, Mendez-Pertuz M, Buscato A, Martinez-Torrecuadrada JL, Blasco MA. Molecular architecture of full-length TRF1 favors its interaction with DNA. *J Biol Chem* 2016; 291: 21829-35
92. Vaurs M, Claude E, Zanella E, et al. TRF1 relies on fork reversal to prevent fragility at human telomeres. *Nat Commun* 2025; 16: 6439
93. Sfeir A, Kosiyatrakul ST, Hockemeyer D, et al. Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell* 2009; 138: 90-103
94. Martínez P, Thanasoula M, Muñoz P, et al. Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. *Genes Dev* 2009; 23: 2060-75
95. Porreca RM, Herrera-Moyano E, Skourti E, et al. TRF1 averts chromatin remodelling, recombination and replication dependent-break induced replication at mouse telomeres. *eLife* 2020; 9: e49817
96. Zimmermann M, Kibe T, Kabir S, Lange T de. TRF1 negotiates TTAGGG repeat-associated replication problems by recruiting the BLM helicase and the TPP1/POT1 repressor of ATR signaling. *Genes Dev* 2014; gad.251611.114
97. Yang Z, Takai KK, Lovejoy CA, de Lange T. Break-induced replication promotes fragile telomere formation. *Genes Dev* 2020; 34: 1392-405
98. Fouquerel E, Barnes RP, Uttam S, Watkins SC, Bruchez MP, Opresko PL. Targeted and persistent 8-oxoguanine base damage at telomeres promotes telomere loss and crisis. *Mol Cell* 2019; 75: 117-130.e6
99. McNees CJ, Tejera AM, Martínez P, et al. ATR suppresses telomere fragility and recombination but is dispensable for elongation of short telomeres by telomerase. *J Cell Biol* 2010; 188: 639-52
100. Smith S, Gariat I, Schmitt A, de Lange T. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* 1998; 282: 1484-7
101. Smith S, de Lange T. Tankyrase promotes telomere elongation in human cells. *Current Biology* 2000; 10: 1299-302
102. Seimiya H, Muramatsu Y, Ohishi T, Tsuruo T. Tankyrase 1 as a target for telomere-directed molecular cancer therapeutics. *Cancer Cell* 2005; 7: 25-37
103. Yang L, Sun L, Teng Y, et al. Tankyrase1-mediated poly(ADP-ribosylation) of TRF1 maintains cell survival after telomeric DNA damage. *Nucleic Acids Res* 2017; 45: 3906-21
104. Chang W, Dynek JN, Smith S. TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres. *Genes Dev* 2003; 17: 1328-33
105. Lee TH, Perrem K, Harper JW, Lu KP, Zhou XZ. The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance. *J Biol Chem* 2006; 281: 759-68

106. Zeng Z, Wang W, Yang Y, et al. Structural basis of selective ubiquitination of TRF1 by SCFFbx4. *Dev Cell* 2010; 18: 214-25
107. Her YR, Chung IK. Ubiquitin ligase RLIM modulates telomere length homeostasis through a proteolysis of TRF1. *J Biol Chem* 2009; 284: 8557-66
108. Kim J, Chung IK. The splicing factor U2AF65 stabilizes TRF1 protein by inhibiting its ubiquitin-dependent proteolysis. *Biochem Biophys Res Commun* 2014; 443: 1124-30
109. Méndez-Pertuz M, Martínez P, Blanco-Aparicio C, et al. Modulation of telomere protection by the PI3K/AKT pathway. *Nat Commun* 2017; 8: 1278
110. Sánchez-Vázquez R, Martínez P, Blasco MA. AKT-dependent signaling of extracellular cues through telomeres impact on tumorigenesis. *PLoS Genet* 2021; 17: e1009410
111. Wu Z-Q, Yang X, Weber G, Liu X. Plk1 phosphorylation of TRF1 is essential for its binding to telomeres. *J Biol Chem* 2008; 283: 25503-13
112. McKerlie M, Walker JR, Mitchell TRH, Wilson FR, Zhu X-D. Phosphorylated (pT371)TRF1 is recruited to sites of DNA damage to facilitate homologous recombination and checkpoint activation. *Nucleic Acids Res* 2013; 41: 10268-82
113. Tong AS, Stern JL, Sfeir A, et al. ATM and ATR signaling regulate the recruitment of human telomerase to telomeres. *Cell Rep* 2015; 13: 1633-46
114. McKerlie M, Lin S, Zhu X-D. ATM regulates proteasome-dependent subnuclear localization of TRF1, which is important for telomere maintenance. *Nucleic Acids Res* 2012; 40: 3975-89
115. Kim MK, Kang MR, Nam HW, Bae Y-S, Kim YS, Chung IK. Regulation of telomeric repeat binding factor 1 binding to telomeres by casein kinase 2-mediated phosphorylation. *J Biol Chem* 2008; 283: 14144-52
116. Tan R, Nakajima S, Wang Q, et al. Nek7 protects telomeres from oxidative DNA damage by phosphorylation and stabilization of TRF1. *Mol Cell* 2017; 65: 818-831.e5
117. Lee TH, Tun-Kyi A, Shi R, et al. Essential role of Pin1 in regulation of TRF1 stability and telomere maintenance. *Nat Cell Biol* 2009; 11: 97-105
118. Ho A, Wilson FR, Peragine SL, Jeyanthan K, Mitchell TRH, Zhu X-D. TRF1 phosphorylation on T271 modulates telomerase-dependent telomere length maintenance as well as the formation of ALT-associated PML bodies. *Sci Rep* 2016; 6: 36913
119. dos Santos GA, Viana NI, Pimenta R, et al. Prognostic value of TERF1 expression in prostate cancer. *J Egypt Natl Canc Inst* 2021; 33: 24
120. Pal D, Sharma U, Singh SK, Kakkar N, Prasad R. Over-expression of telomere binding factors (TRF1 & TRF2) in renal cell carcinoma and their inhibition by using SiRNA induce apoptosis, reduce cell proliferation and migration invitro. *PLoS One* 2015; 10: e0115651
121. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. *Nat Genet* 1999; 23: 405-12
122. Houghtaling BR, Cuttonaro L, Chang W, Smith S. A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. *Curr Biol* 2004; 14: 1621-31
123. Liu D, O'Connor MS, Qin J, Songyang Z. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem* 2004; 279: 51338-42
124. Ye JZ-S, Hockemeyer D, Krutchinsky AN, et al. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. *Genes Dev* 2004; 18: 1649-54
125. Kaminker PG, Kim S-H, Desprez P-Y, Campisi J. A novel form of the telomere-associated protein TIN2 localizes to the nuclear matrix. *Cell Cycle* 2009; 8: 931-9
126. Frescas D, de Lange T. TRF2-tethered TIN2 can mediate telomere protection by TPP1/POT1. *Mol Cell Biol* 2014; 34: 1349-62

127. Takai KK, Kibe T, Donigian JR, Frescas D, de Lange T. Telomere protection by TPP1/POT1 requires tethering to TIN2. *Mol Cell* 2011; 44: 647-59
128. Frescas D, de Lange T. Binding of TPP1 protein to TIN2 protein is required for POT1a,b protein-mediated telomere protection. *J Biol Chem* 2014; 289: 24180-7
129. Abreu E, Aritonovska E, Reichenbach P, et al. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. *Mol Cell Biol* 2010; 30: 2971-82
130. Wang F, Podell ER, Zaug AJ, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature* 2007; 445: 506-10
131. Latrick CM, Cech TR. POT1-TPP1 enhances telomerase processivity by slowing primer dissociation and aiding translocation. *EMBO J* 2010; 29: 924-33
132. Ye JZ-S, de Lange T. TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. *Nat Genet* 2004; 36: 618-23
133. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. *Am J Hum Genet* 2008; 82: 501-9
134. Walne AJ, Vulliamy T, Beswick R, Kirwan M, Dokal I. TINF2 mutations result in very short telomeres: analysis of a large cohort of patients with dyskeratosis congenita and related bone marrow failure syndromes. *Blood* 2008; 112: 3594-600
135. Yang D, Xiong Y, Kim H, et al. Human telomeric proteins occupy selective interstitial sites. *Cell Res* 2011; 21: 1013-27
136. Yang D, He Q, Kim H, Ma W, Songyang Z. TIN2 protein dyskeratosis congenita missense mutants are defective in association with telomerase. *J Biol Chem* 2011; 286: 23022-30
137. Schmutz I, Mensenkamp AR, Takai KK, et al. TINF2 is a haploinsufficient tumor suppressor that limits telomere length. *eLife* 2020; 9: e61235
138. Yin S, Zhang F, Lin S, et al. TIN2 deficiency leads to ALT-associated phenotypes and differentiation defects in embryonic stem cells. *Stem Cell Reports* 2022; 17: 1183-97
139. Canudas S, Houghtaling BR, Kim JY, Dynek JN, Chang WG, Smith S. Protein requirements for sister telomere association in human cells. *EMBO J* 2007; 26: 4867-78
140. Canudas S, Smith S. Differential regulation of telomere and centromere cohesion by the Scc3 homologues SA1 and SA2, respectively, in human cells. *J Cell Biol* 2009; 187: 165-73
141. Canudas S, Houghtaling BR, Bhanot M, et al. A role for heterochromatin protein 1 γ at human telomeres. *Genes Dev* 2011; 25: 1807-19
142. Kaur P, Barnes R, Pan H, et al. TIN2 is an architectural protein that facilitates TRF2-mediated trans- and cis-interactions on telomeric DNA. *Nucleic Acids Res* 2021; 49: 13000-18
143. Liu M, Pan H, Kaur P, et al. Assembly path dependence of telomeric DNA compaction by TRF1, TIN2, and SA1. *Biophys J* 2023; 122: 1822-32
144. Bianchi A, Smith S, Chong L, Elias P, de Lange T. TRF1 is a dimer and bends telomeric DNA. *EMBO J* 1997; 16: 1785-94
145. Bianchi A, Stansel RM, Fairall L, Griffith JD, Rhodes D, de Lange T. TRF1 binds a bipartite telomeric site with extreme spatial flexibility. *EMBO J* 1999; 18: 5735-44
146. Kim S-H, Han S, You Y-H, Chen DJ, Campisi J. The human telomere-associated protein TIN2 stimulates interactions between telomeric DNA tracts in vitro. *EMBO Rep* 2003; 4: 685-91
147. Kalathiya U, Padariya M, Baginski M. Molecular basis and quantitative assessment of TRF1 and TRF2 protein interactions with TIN2 and Apollo peptides. *Eur Biophys J* 2017; 46: 171-87
148. Sekaran V, Soares J, Jarstfer MB. Telomere maintenance as a target for drug discovery. *J Med Chem* 2014; 57: 521-38

149. Tao H, Zhao C, Wang Y, Sheng W, Zhen Y. Targeting telomere dynamics as an effective approach for the development of cancer therapeutics. *IJN* 2024; 19: 3805-25
150. Crunkhorn S. Targeting telomeres. *Nat Rev Drug Discov* 2018; 17: 18-18
151. Ruano JL, Bejarano L, Serrano R, et al. Depletion of the TRF1 telomere-binding protein leads to leaner mice with altered metabolic profiles. *Aging (Albany NY)* 2025; 17: 2410-29
152. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol* 2003; 13: 1549-56
153. Mender I, Shay JW. Telomere dysfunction induced foci (TIF) analysis. *Bio Protoc* 2015; 5: e1656
154. Waksal JA, Bruedigam C, Komrokji RS, Jamieson CHM, Mascarenhas JO. Telomerase-targeted therapies in myeloid malignancies. *Blood Adv* 2023; 7: 4302-14
155. Seimiya H, Smith S. The telomeric poly(ADP-ribose) polymerase, tankyrase 1, contains multiple binding sites for telomeric repeat binding factor 1 (TRF1) and a novel acceptor, 182-kDa tankyrase-binding protein (TAB182). *J Biol Chem* 2002; 277: 14116-26
156. Seimiya H. The telomeric PARP, tankyrases, as targets for cancer therapy. *Br J Cancer* 2006; 94: 341-5
157. Kabir S, Sfeir A, de Lange T. Taking apart Rap1: an adaptor protein with telomeric and non-telomeric functions. *Cell Cycle* 2010; 9: 4061-7
158. Teo H, Ghosh S, Luesch H, et al. Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression. *Nat Cell Biol* 2010; 12: 758-67
159. Martínez P, Gómez-López G, García F, et al. RAP1 protects from obesity through its extratelomeric role regulating gene expression. *Cell Rep* 2013; 3: 2059-74
160. Yeung F, Ramírez CM, Mateos-Gomez PA, et al. Nontelomeric role for Rap1 in regulating metabolism and protecting against obesity. *Cell Rep* 2013; 3: 1847-56
161. Barry RM, Sacco O, Mameri A, et al. Rap1 regulates TIP60 function during fate transition between two-cell-like and pluripotent states. *Genes Dev* 2022; 36: 313-30
162. Martínez P, Gómez-López G, Pisano DG, Flores JM, Blasco MA. A genetic interaction between RAP1 and telomerase reveals an unanticipated role for RAP1 in telomere maintenance. *Aging Cell* 2016; 15: 1113-25
163. Deregowska A, Wnuk M. RAP1/TERF2IP-a multifunctional player in cancer development. *Cancers (Basel)* 2021; 13: 5970
164. Simonet T, Zaragosi L-E, Philippe C, et al. The human TTAGGG repeat factors 1 and 2 bind to a subset of interstitial telomeric sequences and satellite repeats. *Cell Res* 2011; 21: 1028-38
165. Mukherjee AK, Sharma S, Bagri S, et al. Telomere repeat-binding factor 2 binds extensively to extra-telomeric G-quadruplexes and regulates the epigenetic status of several gene promoters. *J Biol Chem* 2019; 294: 17709-22
166. El Maï M, Wagner K-D, Michiels J-F, et al. The telomeric protein TRF2 regulates angiogenesis by binding and activating the PDGFR β promoter. *Cell Rep* 2014; 9: 1047-60
167. Zizza P, Dinami R, Porru M, et al. TRF2 positively regulates SULF2 expression increasing VEGF-A release and activity in tumor microenvironment. *Nucleic Acids Res* 2019; 47: 3365-82
168. Sharma S, Mukherjee AK, Roy SS, et al. Human telomerase is directly regulated by non-telomeric TRF2-G-quadruplex interaction. *Cell Rep* 2021; 35: 109154
169. Hussain T, Saha D, Purohit G, et al. Transcription regulation of CDKN1A (p21/CIP1/WAF1) by TRF2 is epigenetically controlled through the REST repressor complex. *Sci Rep* 2017; 7: 11541
170. Mayayo-Peralta I, Debets DO, Prekovic S, et al. Proteomics on malignant pleural effusions reveals ER α loss in metastatic breast cancer associates with SGK1-NDRG1 deregulation. *Mol Oncol* 2024; 18: 156-69

171. Purohit G, Mukherjee AK, Sharma S, Chowdhury S. Extratelomeric binding of the telomere binding protein TRF2 at the PCGF3 promoter is G-quadruplex motif-dependent. *Biochemistry* 2018; 57: 2317-24
172. Biroccio A, Cherfils-Vicini J, Augereau A, et al. TRF2 inhibits a cell-extrinsic pathway through which natural killer cells eliminate cancer cells. *Nat Cell Biol* 2013; 15: 818-28
173. Cherfils-Vicini J, Iltis C, Cervera L, et al. Cancer cells induce immune escape via glycocalyx changes controlled by the telomeric protein TRF2. *EMBO J* 2019; 38: EMBJ2018100012
174. Mao Z, Seluanov A, Jiang Y, Gorbunova V. TRF2 is required for repair of nontelomeric DNA double-strand breaks by homologous recombination. *Proc Natl Acad Sci U S A* 2007; 104: 13068-73
175. Huda N, Abe S, Gu L, Mendonca MS, Mohanty S, Gilley D. Recruitment of TRF2 to laser-induced DNA damage sites. *Free Radic Biol Med* 2012; 53: 1192-7
176. Mendez-Bermudez A, Lototska L, Bauwens S, et al. Genome-wide control of heterochromatin replication by the telomere capping protein TRF2. *Mol Cell* 2018; 70: 449-461.e5
177. Mukherjee AK, Sharma S, Sengupta S, et al. Telomere length-dependent transcription and epigenetic modifications in promoters remote from telomere ends. *PLoS Genet* 2018; 14: e1007782
178. Sengupta A, Vinayagamurthy S, Soni D, et al. Telomeres control human telomerase (TERT) expression through non-telomeric TRF2. *eLife* 2025; 14: RP104045
179. Schrödinger L. Maestro [Internet]. New York, NY, USA: Schrödinger, LLC; 2021. Available from: <https://www.schrodinger.com/citations/>
180. Schrödinger L. The PyMOL Molecular Graphics System [Internet]. New York, NY, USA: Schrödinger, LLC; Available from: <https://www.pymol.org/>
181. Dasmahapatra U, Kumar CK, Das S, et al. In-silico molecular modelling, MM/GBSA binding free energy and molecular dynamics simulation study of novel pyrido fused imidazo[4,5-c]quinolines as potential anti-tumor agents. *Front Chem* 2022; 10: 991369
182. Riss TL, Moravec RA, Niles AL, et al. Cell viability assays. In: Markossian S, Grossman A, Baskir H, et al., editors. *Assay Guidance Manual* [Internet] Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004 [cited 2026 Aug 1]. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK144065/>
183. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. Springer-Verlag New York; 2016. Available from: <https://ggplot2.tidyverse.org>
184. GraphPad Software, LLC. GraphPad Prism for Windows [Internet]. Boston, MA: GraphPad Software, LLC; 2025. Available from: <https://www.graphpad.com/>
185. Holme B, Bjørnerud B, Pedersen NM, de la Ballina LR, Wesche J, Haugsten EM. Automated tracking of cell migration in phase contrast images with CellTraxx. *Sci Rep* 2023; 13: 22982
186. Cytiva. Biacore T200 Operating Instructions [Internet]. Uppsala, Sweden: Cytiva; Available from: <https://www.cytivalifesciences.com/en/us/products/items/biacore-t200-p-05644>
187. Cossarizza A, Chang H-D, Radbruch A, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol* 2021; 51: 2708-3145
188. Kim KH, Sederstrom JM. Assaying cell cycle status using flow cytometry. *Curr Protoc Mol Biol* 2015; 111: 28.6.1-28.6.11
189. Bhattacharya D, Nagaraju G. A quantitative DNA fiber assay to monitor replication fork progression, protection, and restart. *Bio Protoc* 2026; 16: e5593
190. Geraud M, Martinez LF, Ajello AC, Cristini A, Sordet O. Protocol for single-cell analysis of DNA double-strand break production and repair in cell-cycle phases by automated high-content microscopy. *STAR Protoc* 2025 [cited 2026 Aug 1]; 6 Available from: [https://www.cell.com/star-protocols/abstract/S2666-1667\(25\)00068-1](https://www.cell.com/star-protocols/abstract/S2666-1667(25)00068-1)

191. Sivandzade F, Bhalerao A, Cucullo L. Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe. *Bio Protoc* 2019; 9: e3128
192. Telford WG. Multiparametric analysis of apoptosis by flow cytometry. *Methods Mol Biol* 2018; 1678: 167-202
193. Im K, Mareninov S, Diaz MFP, Yong WH. An introduction to performing immunofluorescence staining. *Methods Mol Biol* 2019; 1897: 299-311
194. Thermo Fisher Scientific. Invitrogen CellEvent Senescence Green Detection Kit [Internet]. Waltham, Massachusetts, USA: Thermo Fisher Scientific; [cited 2026 Aug 1]. Available from: <https://www.thermofisher.com/order/catalog/product/C10850>
195. Adewoye AB, Tampakis D, Follenzi A, Stolzing A. Multiparameter flow cytometric detection and quantification of senescent cells in vitro. *Biogerontology* 2020; 21: 773-86
196. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc* 2006; 1: 2315-9
197. Leary E, Curran S, Susienka M, Manning KL, Blakely AM, Morgan JR. Micro-moulded non-adhesive hydrogels to form multicellular microtissues - the 3D Petri Dish®. *Technology Platforms for 3D Cell Culture* [Internet] John Wiley & Sons, Ltd; 2017 [cited 2026 Aug 1]. p. 97-122 Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118851647.ch5>
198. Martinotti S, Ranzato E. Scratch wound healing assay. *Methods Mol Biol* 2020; 2109: 225-9
199. Chastel T, Filiberti S, Mitola S, Ronca R, Turtoi A, Corsini M. Protocol for performing angiogenic and tumorigenic assays using the in ovo chick embryo chorioallantoic membrane model. *STAR Protoc* 2025; 6: 103663
200. Thomé MP, Filippi-Chiela EC, Villodre ES, et al. Ratiometric analysis of Acridine Orange staining in the study of acidic organelles and autophagy. *J Cell Sci* 2016; 129: 4622-32
201. Cesare AJ, Heaphy CM, O'Sullivan RJ. Visualization of telomere integrity and function in vitro and in vivo using immunofluorescence techniques. *Curr Protoc Cytom* 2015; 73: 12.40.1-12.4031
202. Baerlocher GM, Mak J, Tien T, Lansdorp PM. Telomere length measurement by fluorescence in situ hybridization and flow cytometry: tips and pitfalls. *Cytometry* 2002; 47: 89-99
203. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2025. Available from: <https://www.R-project.org/>
204. Posit team. RStudio: Integrated Development Environment for R [Internet]. Boston, MA: Posit Software, PBC; 2025. Available from: <https://posit.co/>
205. Becton, Dickinson and Company. FlowJo Software for Windows [Internet]. Ashland, OR: Becton, Dickinson and Company; 2023. Available from: <https://flowjo.com/citing-flowjo>
206. Agilent Technologies Inc. NovoExpress Software [Internet]. Santa Clara, California, USA: Agilent Technologies, Inc.; [cited 2026 July 31]. Available from: <https://www.agilent.com/en/product/research-flow-cytometry/flow-cytometry-software/novocyte-novoexpress-software-1320805>
207. Bio-Rad Laboratories Inc. Image Lab Software [Internet]. Hercules, California, USA: Bio-Rad Laboratories, Inc.; 2014. Available from: <https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z>
208. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; 9: 671-5
209. Evident Corporation. cellSens Imaging Software [Internet]. Tokyo, Japan: Evident Corporation; [cited 2026 July 31]. Available from: <https://evidentscientific.com/en/products/software/cellsens>
210. Cytiva. Biacore T200 Evaluation Software [Internet]. Uppsala, Sweden: Cytiva; 2021. Available from:

- <https://www.cytivalifesciences.com/en/us/support/software/biacore-downloads/biacore-t200-software>
211. Bhojwani A, Little T, Hyde C, Ventura T. nf-core/denovotranscript: a workflow for de novo transcriptome assembly and quantification of paired-end short reads from bulk RNA-seq. *Curr Protoc* 2025; 5: e70248
 212. Friesner RA, Banks JL, Murphy RB, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004; 47: 1739-49
 213. Sastry GM, Adzhigirey M, Day T, Annabhimoju R, Sherman W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. *J Comput Aided Mol Des* 2013; 27: 221-34
 214. Bowers KJ, Sacerdoti FD, Salmon JK, et al. Molecular dynamics---Scalable algorithms for molecular dynamics simulations on commodity clusters. *Proceedings of the 2006 ACM/IEEE conference on Supercomputing - SC '06* Tampa, Florida: ACM Press; 2006; 84
 215. Fumagalli M, Rossiello F, Clerici M, et al. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nat Cell Biol* 2012; 14: 355-65
 216. Freund A, Laberge R-M, Demaria M, Campisi J, Magin TM. Lamin B1 loss is a senescence-associated biomarker. *Mol Biol Cell* 2012; 23: 2066-75
 217. Sahin E, Colla S, Liesa M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011; 470: 359-65
 218. Jänicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998; 273: 9357-60
 219. Imamura Y, Mukohara T, Shimono Y, et al. Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncology Reports* 2015; 33: 1837-43

Appendix

Appendix 1. SPR data processing and calculation of binding parameters

Surface plasmon resonance (SPR) sensorgrams were analysed to characterise the interaction of the tested compounds with immobilised TRF1. Compound concentrations were assigned according to the order of the X/Y sensorgram trace pairs in the source files.

SPR response (RU)

The response unit (RU) represents the SPR signal associated with analyte accumulation at the sensor surface. For each concentration, RU was obtained from the stable association plateau. To avoid the valve switch transition immediately before dissociation, the final 5 s were excluded and the median response during the preceding 10 s was calculated:

$$RU_i = \text{median}[R_i(t)]_{t_{\text{diss}}-15\text{ s}}^{t_{\text{diss}}-5\text{ s}}$$

where $R_i(t)$ is the recorded response for concentration i and t_{diss} is the beginning of the dissociation phase. The value reported in the final table corresponds to the highest observed response among the non-zero concentrations:

$$RU_{\text{max,obs}} = \max(RU_1, RU_2, \dots, RU_n)$$

Thus, the reported RU is an observed response and not a fitted R_{max} .

Association rate constant (k_a)

For a simple 1:1 interaction, the association phase may be described by:

$$R(t) = B + A(1 - e^{-k_{\text{obs}}t})$$

where k_{obs} is the observed association rate at a given analyte concentration. Its concentration dependence is:

$$k_{\text{obs}} = k_a C + k_d$$

Accordingly, k_a is obtained from the slope of k_{obs} versus analyte concentration:

$$k_a = \frac{\Delta k_{\text{obs}}}{\Delta C}$$

When concentration is expressed in nM, k_a is expressed in $\text{nM}^{-1} \text{s}^{-1}$. A larger k_a indicates faster association.

Dissociation rate constant (k_d)

The dissociation phase is described by exponential decay:

$$R(t) = C_0 + Ae^{-k_d t}$$

where k_d is the dissociation rate constant in s^{-1} . A smaller k_d indicates slower dissociation and a more persistent analyte protein complex.

Kinetic dissociation constant (K_D)

When physically valid k_a and k_d values are available, the kinetic dissociation constant is calculated as:

$$K_D^{\text{kinetic}} = \frac{k_d}{k_a}$$

Using k_a in $\text{nM}^{-1} \text{s}^{-1}$ and k_d in s^{-1} gives K_D in nM. A lower K_D indicates higher apparent affinity. Non-positive k_a or k_d values are physically invalid and were therefore reported as NA. If either rate constant was unavailable, the corresponding kinetic K_D was also reported as NA.

The equilibrium association constant, where required, can be obtained from:

$$K_A = \frac{1}{K_D}$$

Thus, unlike K_D , a larger K_A indicates stronger binding.

Steady state K_D

Steady state K_D is determined independently of k_a and k_d by fitting the concentration dependent equilibrium response to a 1:1 binding isotherm:

$$R_{\text{eq}} = \frac{R_{\text{max}}C}{K_D + C}$$

where R_{eq} is the equilibrium response, C is analyte concentration and R_{max} is the fitted maximum response. Therefore, a steady state K_D can still be obtained when kinetic rate constants cannot be resolved.

When a clear binding plateau was not reached within the tested concentration range, the fitted steady state K_D was considered a mathematical estimate and marked with ** in the results table: *The steady state K_D was mathematically derived without a clear binding plateau and should be interpreted with caution.*

Comparison and limitations

Affinity was compared primarily using valid K_D values, with lower K_D indicating higher apparent affinity. The k_a and k_d values describe the rates of association and dissociation, respectively, whereas RU reflects the magnitude of the observed SPR response and should not be used alone for affinity ranking.

Some sensorgrams showed rapid or step like association and dissociation, limiting reliable kinetic fitting. In addition, several compounds did not reach clear saturation within the tested concentration range, resulting in very large mathematically fitted steady state K_D values. Large disagreement between kinetic and steady state K_D values was therefore interpreted cautiously.

Raw data availability

The original SPR concentration series data and associated analysis files used for these calculations are available in the electronic Drive accompanying this thesis for independent inspection and re-analysis.

Table 1. Cell lines used in the study, tissue origin, and routine culture conditions

Cell line	Tissue / origin	Culture medium	CO ₂ condition	Special notes
A549	Lung adenocarcinoma	RPMI	5% CO ₂	-
HCT116	Colorectal carcinoma	McCoy's	5% CO ₂	-
HeLa	Cervical carcinoma	MEM	5% CO ₂	-
MCF7	Breast adenocarcinoma	RPMI	5% CO ₂	-
HepG2	Hepatocellular carcinoma	MEM	5% CO ₂	-
U-2 OS	Osteosarcoma	McCoy's	5% CO ₂	-
A2780	Ovarian carcinoma	RPMI	5% CO ₂	-
U-87 MG	Glioblastoma	MEM	5% CO ₂	-
HEK-293	Human embryonic kidney	DMEM	10% CO ₂	-
HaCaT	Immortalised human keratinocyte	RPMI	5% CO ₂	-
MRC-5	Human lung fibroblast	EMEM	5% CO ₂	-
BT-474	Breast ductal carcinoma	Hybri-Care medium	5% CO ₂	10% FBS
HT29-MTX	Colorectal adenocarcinoma	McCoy's	5% CO ₂	-
MDA-MB-231	Breast adenocarcinoma	DMEM	10% CO ₂	-
SKBR3	Breast adenocarcinoma	DMEM	10% CO ₂	-
T-47D	Breast ductal carcinoma	DMEM	10% CO ₂	-
HT29	Colorectal adenocarcinoma	McCoy's	5% CO ₂	-
LoVo	Colorectal adenocarcinoma	F12K	5% CO ₂	-
DU145	Prostate carcinoma	EMEM	5% CO ₂	-
CCD 841 CoN	Normal colon epithelial	EMEM	5% CO ₂	-
BT-20	Breast carcinoma	RPMI	5% CO ₂	-
MCF7 Tam1	Breast adenocarcinoma	RPMI	5% CO ₂	1 µM 4-hydroxytamoxifen and 10 µg/mL human insulin
LNCaP	Prostate adenocarcinoma	RPMI	5% CO ₂	-
HMEC	Normal human mammary epithelium	Mammary Epithelial Cell Basal Medium	5% CO ₂	Supplemented with all components of the Mammary Epithelial Cell Growth Kit

Note. Unless otherwise stated, cells were maintained at 37 °C in a humidified incubator and cultured in their respective media supplemented with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (62.6 µg/mL), and streptomycin (40 µg/mL). Cell cultures were routinely monitored for mycoplasma contamination and morphological abnormalities. BT-474 and HMEC culture conditions were completed according to ATCC recommendations.

Abbreviations: CO₂, carbon dioxide; FBS, foetal bovine serum.

Table 2. IC50 values of the compounds and standard drugs

Cell line	TRFiN 45	TRFiN 47	TRFiN 33	TRFiN 44	TRFiN 72	TRFiN 67b	TRFiN 41	TRFiN 49	TRFiN 38	TRFiN 42	TRFiN 43	A822	4OHT	ETP
MCF7	19.46 ± 1.23	11.19 ± 0.92	33.86 ± 3.28	17.53 ± 1.66	7.62 ± 0.69	8.27 ± 0.42	11.71 ± 0.40	5.96 ± 0.69	9.85 ± 1.14	11.77 ± 1.00	28.30 ± 1.64	4.80 ± 0.65	1.98 ± 0.51	20.07 ± 1.66
BT-474	53.82 ± 1.66	22.37 ± 0.24	21.14 ± 0.77	46.77 ± 0.41	8.46 ± 0.69	8.74 ± 0.95	27.18 ± 1.29	12.31 ± 0.31	18.39 ± 1.62	19.92 ± 1.06	8.02 ± 0.62	9.41 ± 1.37	6.16 ± 1.25	21.29 ± 1.94
MDA-M B-231	56.25 ± 5.13	21.32 ± 3.72	7.92 ± 7.23	52.41 ± 1.41	6.17 ± 0.57	6.64 ± 0.41	22.65 ± 2.71	11.96 ± 0.60	16.63 ± 1.76	18.56 ± 1.93	63.54± 32.21	7.62 ± 1.66	2.78 ± 1.39	17.00 ± 0.58
SKBR3	41.59 ± 4.79	15.15 ± 1.03	37.65 ± 4.58	38.69 ± 8.10	6.10 ± 0.49	7.68 ± 0.46	16.10 ± 1.23	9.95 ± 0.65	12.32 ± 1.14	14.35 ± 0.60	42.40 ± 6.90	10.20 ± 1.85	3.35 ± 2.13	7.10 ± 1.65
T-47D	25.54 ± 8.38	8.66 ± 0.49	21.88 ± 7.60	28.03 ± 1.31	4.05 ± 0.23	4.51 ± 0.48	9.98 ± 0.96	6.81 ± 0.27	8.37 ± 0.77	8.57 ± 0.61	25.57 ± 6.61	5.04 ± 1.30	2.42 ± 0.82	7.87 ± 0.69
BT-20	29.90 ± 6.33	24.16 ± 1.01	7.98 ± 4.05	34.88 ± 1.68	9.74 ± 0.80	9.63 ± 0.71	23.95 ± 0.59	11.37 ± 0.81	19.42 ± 1.15	21.64 ± 1.79	51.16 ± 1.58	14.69 ± 0.51	14.72 ± 0.73	7.27 ± 0.84
MCF7 Tam1	28.85 ± 1.10	14.33 ± 2.29	4.79 ± 1.38	15.30 ± 1.97	5.97 ± 0.69	6.64 ± 0.86	13.67 ± 1.06	8.55 ± 0.37	12.97 ± 1.01	11.76 ± 1.46	18.85 ± 1.80	13.73 ± 2.31	2.30 ± 0.30	21.84 ± 1.27
HMEC	23.43 ± 0.12	34.89 ± 1.68	11.92 ± 1.50	20.65 ± 9.11	31.94 ± 1.75	13.55 ± 2.17	40.87 ± 4.08	30.51 ± 1.30	42.22 ± 1.89	37.86 ± 2.22	15.06 ± 0.37	4.91 ± 0.02	3.34 ± 0.64	25.25 ± 1.53
HCT116	28.35 ± 1.14	13.06 ± 0.79	42.21 ± 2.13	31.58 ± 2.49	8.37 ± 1.53	9.43 ± 1.59	12.83 ± 1.17	6.88 ± 0.25	12.64 ± 1.26	13.38 ± 0.64	37.03 ± 1.58	4.63 ± 0.61	2.64 ± 0.46	24.71 ± 0.60
HT29	41.88 ± 1.14	21.61 ± 0.36	54.57 ± 1.25	44.94 ± 1.28	5.51 ± 0.36	6.21 ± 0.62	28.22 ± 2.89	9.50 ± 0.41	12.52 ± 1.39	19.39 ± 1.29	57.12 ± 2.69	5.68 ± 0.34	2.34 ± 0.33	5.79 ± 0.32
HT29-M TX	23.65 ± 1.93	13.86 ± 0.90	31.55 ± 1.88	25.48 ± 1.72	5.82 ± 1.25	7.06 ± 1.12	15.02 ± 0.26	7.73 ± 1.90	17.91 ± 0.96	18.56 ± 0.33	40.26 ± 1.37	6.35 ± 0.63	2.64 ± 0.019	18.76 ± 0.03
LoVo	41.99 ± 1.71	26.95 ± 0.25	41.72 ± 1.53	34.94 ± 1.11	9.67 ± 0.97	12.53 ± 0.79	41.22 ± 2.14	16.63 ± 1.47	31.11 ± 0.49	38.98 ± 1.92	23.45 ± 1.09	9.41 ± 0.95	3.04 ± 0.28	18.96 ± 1.25
CCD 841 CoN	25.00 ± 1.14	14.88 ± 0.92	15.44 ± 0.36	24.43 ± 0.90	13.70 ± 1.38	11.52 ± 1.72	16.56 ± 0.98	11.94 ± 0.96	12.71 ± 0.47	8.69 ± 0.74	15.42 ± 0.20	0.61 ± 0.013	2.87 ± 0.053	11.57 ± 0.42
A549	30.17 ± 1.14	12.83 ± 0.25	44.56 ± 1.19	30.35 ± 0.04	7.87 ± 0.51	10.27 ± 0.13	14.02 ± 0.79	6.93 ± 0.40	11.59 ± 0.55	12.04 ± 0.67	51.42 ± 1.23	3.54 ± 0.80	3.15 ± 0.23	18.74 ± 1.38
HeLa	35.21 ± 2.95	19.49 ± 0.98	42.12 ± 1.95	36.47 ± 2.92	9.95 ± 0.03	11.53 ± 1.32	12.57 ± 0.77	8.60 ± 0.60	16.01 ± 0.30	16.64 ± 1.79	34.15 ± 1.31	5.75 ± 0.89	2.11 ± 0.11	5.80 ± 0.021
HepG2	29.88 ± 0.96	14.51 ± 0.60	10.55 ± 0.21	26.79 ± 1.12	4.16 ± 0.20	9.85 ± 1.16	17.39 ± 1.33	6.48 ± 0.19	14.35 ± 0.34	15.15 ± 0.52	52.17 ± 3.97	6.17 ± 1.83	2.59 ± 0.051	7.93 ± 0.45
U-2 OS	31.58 ± 2.79	15.62 ± 1.72	43.72 ± 2.50	34.02 ± 0.96	4.48 ± 0.84	8.81 ± 0.26	16.08 ± 0.65	7.57 ± 0.12	14.14 ± 0.45	15.28 ± 0.07	50.52 ± 1.63	9.85 ± 1.04	2.57 ± 0.016	7.54 ± 0.024
A2780	22.26 ± 1.75	7.30 ± 0.17	19.07 ± 1.38	19.79 ± 1.58	3.33 ± 0.12	7.80 ± 0.28	9.62 ± 0.92	5.47 ± 0.37	8.81 ± 0.43	8.79 ± 0.41	20.50 ± 2.87	8.38 ± 0.60	1.82 ± 0.17	6.30 ± 0.13
U-87 MG	37.52 ± 2.63	21.13 ± 1.13	5.33 ± 0.51	44.78 ± 2.26	6.12 ± 0.34	14.75 ± 0.63	23.32 ± 1.20	10.01 ± 0.36	18.26 ± 1.59	17.33 ± 0.09	46.39 ± 1.34	4.93 ± 0.57	1.92 ± 0.045	11.57 ± 0.85
DU145	39.80 ± 6.86	21.21 ± 4.05	30.46± 14.86	42.38 ± 7.48	5.80 ± 1.09	6.43 ± 1.08	20.31 ± 1.89	7.91 ± 0.66	17.67 ± 8.95	17.10 ± 2.23	27.58 ± 1.10	9.21 ± 0.83	6.08 ± 0.40	15.01 ± 1.01
LNCaP	34.41 ± 2.13	15.83 ± 1.05	6.57 ± 0.66	39.61 ± 1.09	6.40 ± 0.30	7.04 ± 0.50	15.87 ± 1.68	8.92 ± 0.14	18.31 ± 1.63	14.37 ± 1.46	13.28 ± 2.08	1.11 ± 0.010	4.61 ± 0.25	3.28 ± 0.044
MRC-5	19.96 ± 1.10	9.89 ± 0.33	43.86 ± 3.72	28.08 ± 1.98	3.37 ± 0.08	8.03 ± 0.23	13.46 ± 0.69	5.39 ± 0.43	10.04 ± 0.96	11.80 ± 1.02	54.61 ± 2.16	16.55 ± 0.22	10.15 ± 0.84	6.63 ± 0.31
HaCaT	27.53 ± 1.08	11.79 ± 1.44	34.78 ± 8.76	23.14 ± 0.84	3.89 ± 0.049	9.13 ± 1.35	13.81 ± 0.27	7.03 ± 0.54	10.81 ± 0.75	11.69 ± 0.63	38.22 ± 1.02	7.47 ± 1.51	1.90 ± 0.38	7.45 ± 0.32
HEK-293	24.85 ± 1.39	9.81 ± 1.06	24.86 ± 1.78	26.88 ± 1.37	2.91 ± 0.10	8.65 ± 1.24	9.16 ± 0.55	5.44 ± 0.10	8.77 ± 0.48	8.83 ± 0.63	44.01 ± 3.63	6.58 ± 1.78	2.56 ± 0.061	5.87 ± 0.36

Note. Data are presented as mean ± SD (n = 3).

Table 3. *List of antibodies and fluorophores used*

<i>Name</i>	<i>Vendor & catalogue number</i>	<i>Dilution</i>
Lamin B1	Abcam, ab133741	1:1000 (WB)
p21 (CDKN1A)	Santa cruz, sc-397	1:500(WB)
p27 Kip1 (CDKN1B)	Santa cruz, sc-1641	1:500 (WB)
p16 (CDKN2A)	Cell signalling, 80772	1:1000 (WB)
Cyclin D1	Cell signalling, 2926	1:1000 (WB)
Cyclin E1	Santa cruz, sc-247	1:1000 (WB)
γ H2AX (phospho histone H2AX, Ser139)	Abcam, ab26350	1:1000 (WB)
Phospho-CHK1 (pCHK1)	Cell signalling, sc-5345	1:1000 (WB)
CHK1 (total)	Cell signalling, sc-2360	1:1000 (WB)
Phospho-CHK2 (pCHK2)	Cell signalling, sc-2661	1:1000 (WB)
CHK2 (total)	Cell signalling, sc-3440	1:1000 (WB)
Phospho ATM (pATM)	Cell signalling, sc-5883	1:1000 (WB)
ATM (total)	Abcam, ab78	1:1000 (WB)
Phospho ATR (pATR)	Cell signalling, sc-2853	1:1000 (WB)
ATR (total)	Abcam, ab10312	1:1000 (WB)
Bcl-2	Santa cruz, sc-509	1:1000 (WB)
BAX	Cell signalling, 2772	1:1000 (WB)
PUMA (BBC3)	Abcam, ab9643	1:800 (WB)
Cytochrome c	Abcam, ab110325	1:1000 (WB)
Apoptosis inducing factor (AIF)	Cell signalling, 4642	1:1000 (WB)
PARP	Cell signalling, 9542	1:1000 (WB)
Caveolin-1	Abcam, ab2910	1:700 (WB)
RhoA	Abcam, ab54835	1:1000 (WB)
TRF1/2	Abcam, ab10579	1:1000 (WB)
E-cadherin	Abcam, ab40772	1:1000 (WB)
N-cadherin	Cell signalling, 13116	1:1000 (WB)
β actin (loading control)	Santa cruz, sc-1616	1:1200 (WB)
TIN2	Abcam, ab197894	1:1000 (WB)
γ H2AX (Alexa Fluor 488-conjugated)	BD Biosciences	1:300 (IF)
TOMM20	Abcam, ab209606	1:1000 (WB)
Anti-BrdU, mouse monoclonal (detects IdU)	Becton Dickinson, 347580	1:100 (IF, DNA fibre)
Anti-BrdU, rat monoclonal (detects CldU)	Abcam, ab6326	1:100 (IF, DNA fibre)
Anti-BrdU, (Alexa Fluor 488-conjugated)	Abcam, ab220074	1:150 (flow cytometry)
Anti-H2AX, Alexa Fluor 488-conjugated	BD Biosciences	1:200 (flow cytometry)

SECONDARY ANTIBODIES		
Goat anti-mouse IgG, Alexa Fluor 488	Santa cruz	1:200 (IF)
Goat anti-rat IgG, Alexa Fluor 594	Santa cruz	1:200 (IF)
Anti-rabbit IgG, HRP conjugated	Jackson ImmunoResearch	- (WB)
Anti-mouse IgG, HRP conjugated	Jackson ImmunoResearch	- (WB)
FLUOROPHORES, PROBES AND STAINS		
Telomere PNA probe, Alexa Fluor 647 labelled	HLB Panagene	0.3 µg/mL (Telo-FISH, Flow-FISH)
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich	1 µg/mL (IF); 0.5 µg/mL (FISH)
Hoechst 33342	Sigma-Aldrich	0.5 µg/mL
Propidium iodide (PI)	Sigma-Aldrich	23 µg/mL
7-Aminoactinomycin D (7-AAD)	Cayman Chemical	3.3 µM; 0.25 µL per sample
FITC-Annexin V	Thermo Fisher Scientific, V13242	0.25 µL per sample
CellEvent Caspase-3/7 Green detection reagent	Thermo Fisher Scientific, C10427	0.25 µL per sample
CellEvent Senescence Green probe	Invitrogen	0.34 µL per sample
JC-1	Sigma-Aldrich	5 µg/mL
MitoTracker Green	Thermo Fisher Scientific	0.5 µM
CellTracker Green	Invitrogen	50 µM
Phalloidin (F actin stain)	Invitrogen	0.5 µL per sample
Acridine orange	Sigma-Aldrich	3 µM
X-Gal	Sigma-Aldrich	1 mg/mL
Crystal violet	Sigma-Aldrich	0.5% (w/v)

Note. (WB) dilution used for western blotting; (IF) dilution used for immunofluorescence

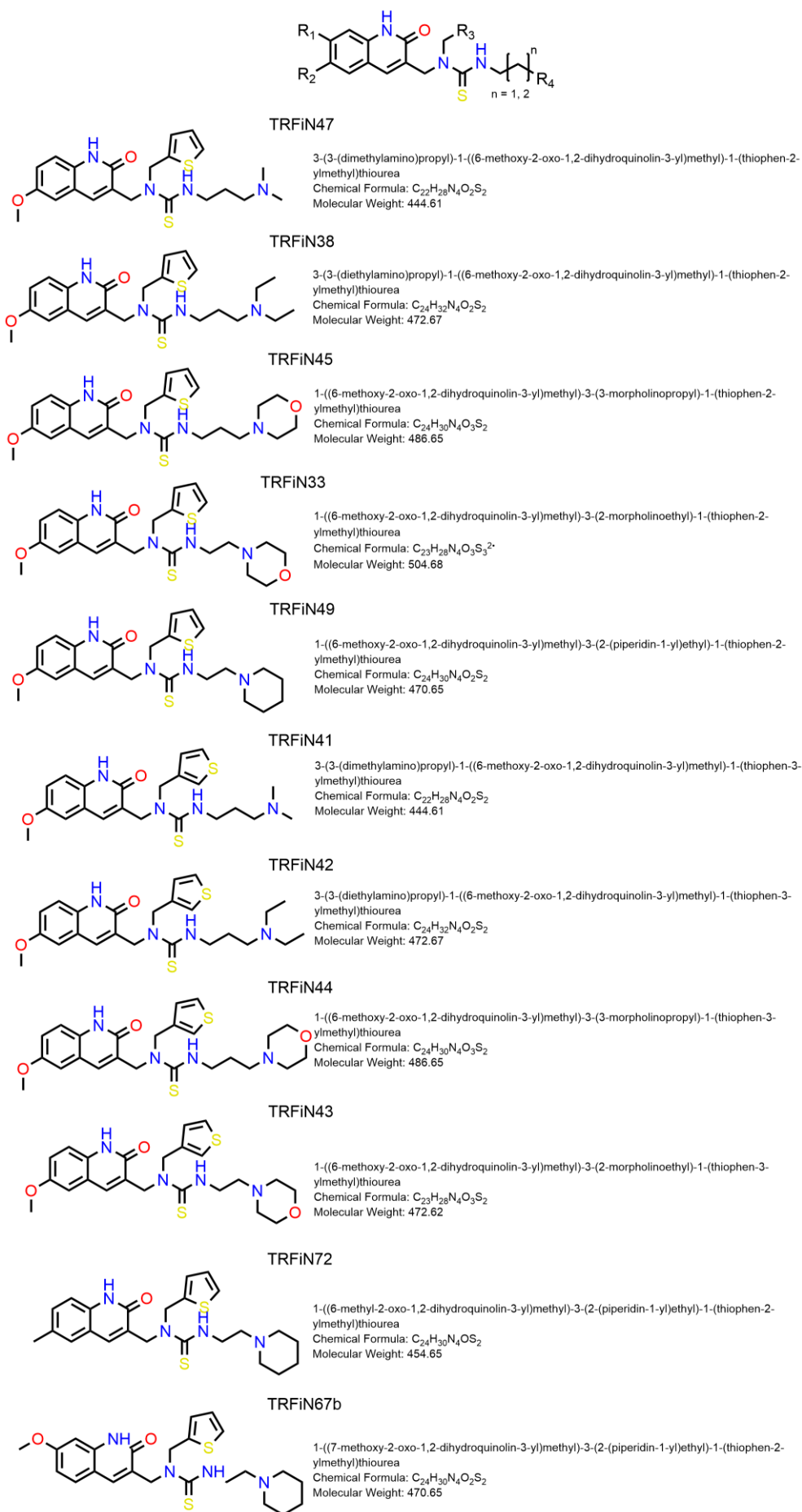
Table 4. Structure of novel A822 derivatives (TRFiN series)

Figure 1. Raw data of western blot (full blots)

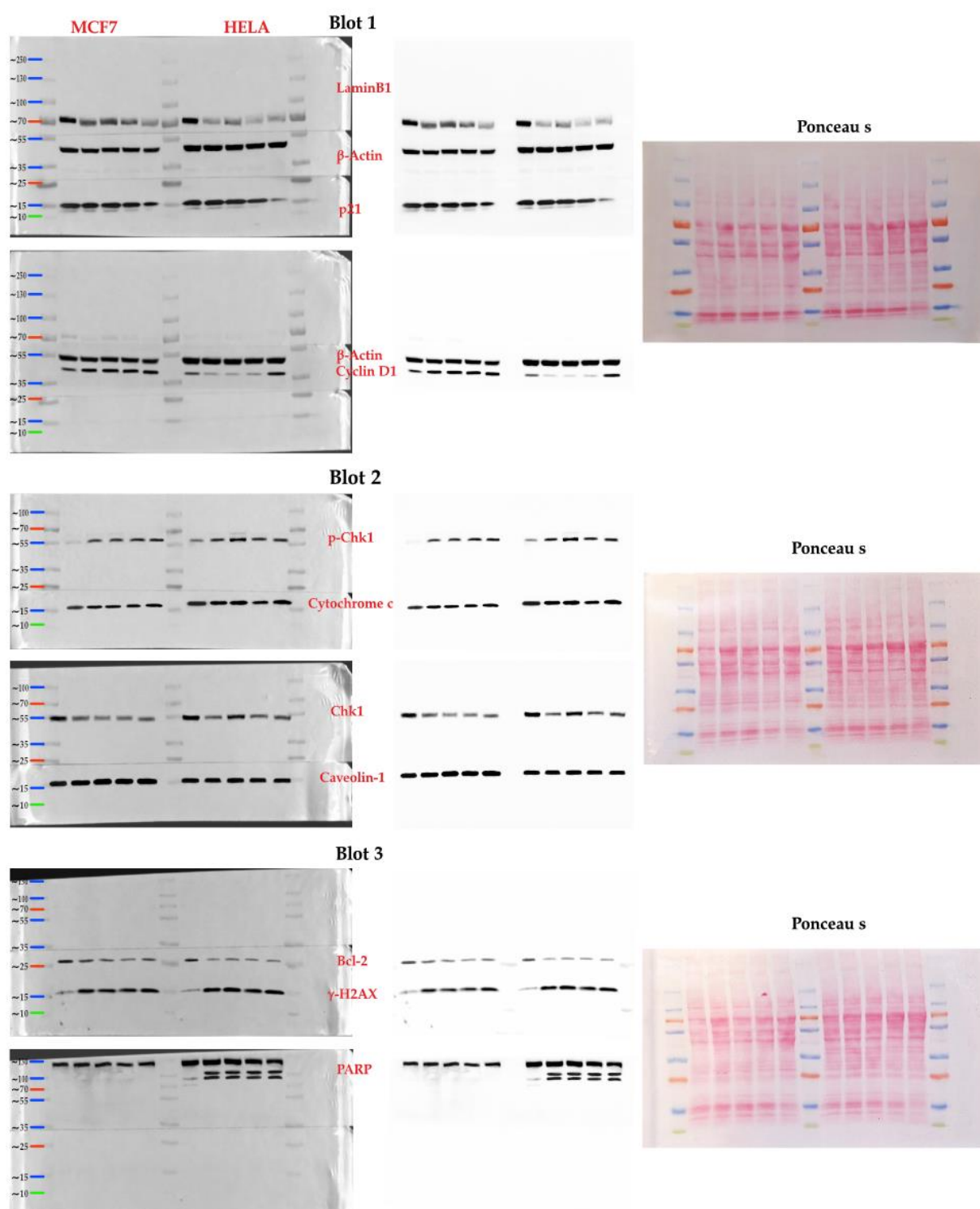


Figure 2. Raw data of western blot (full blots)

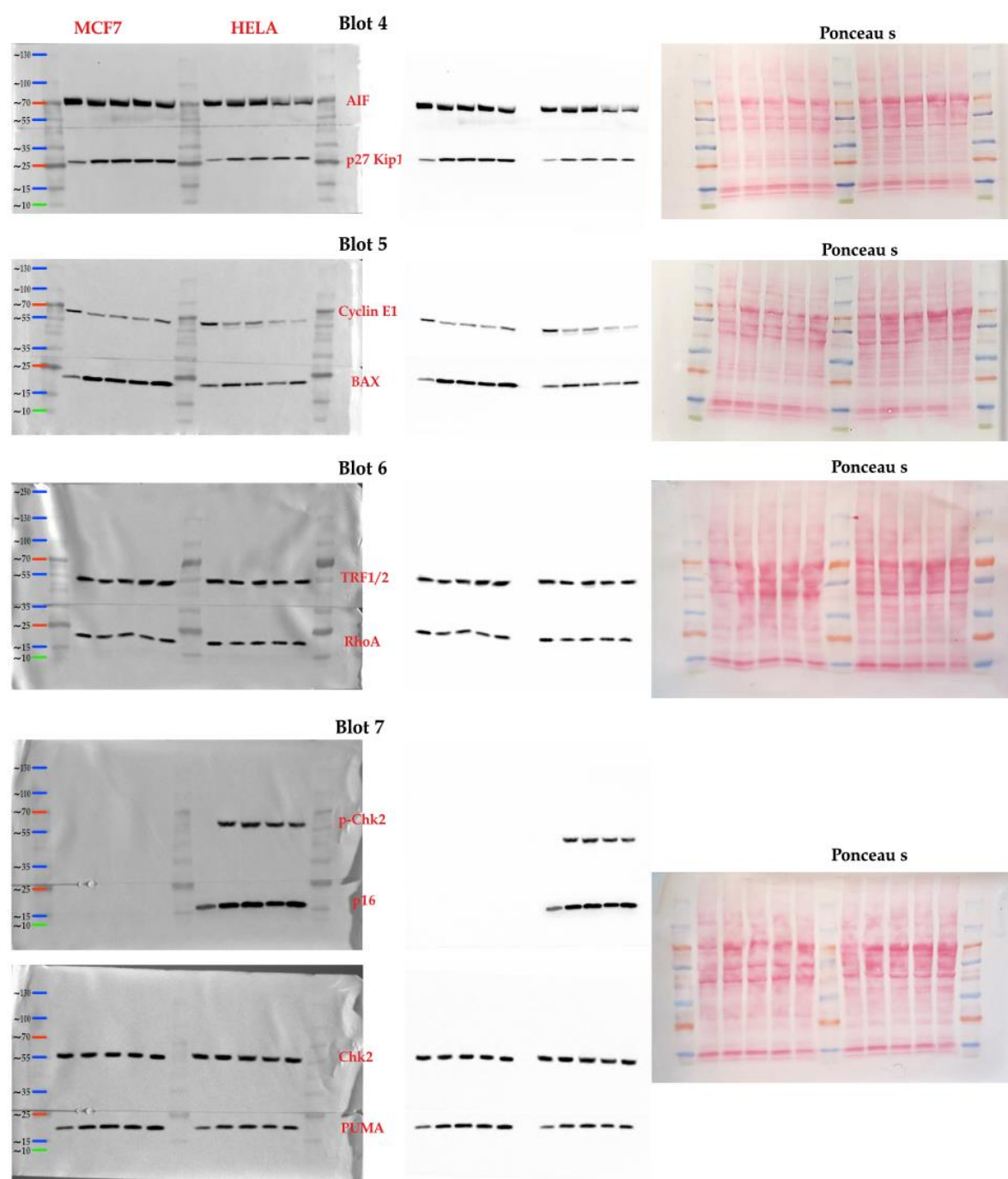


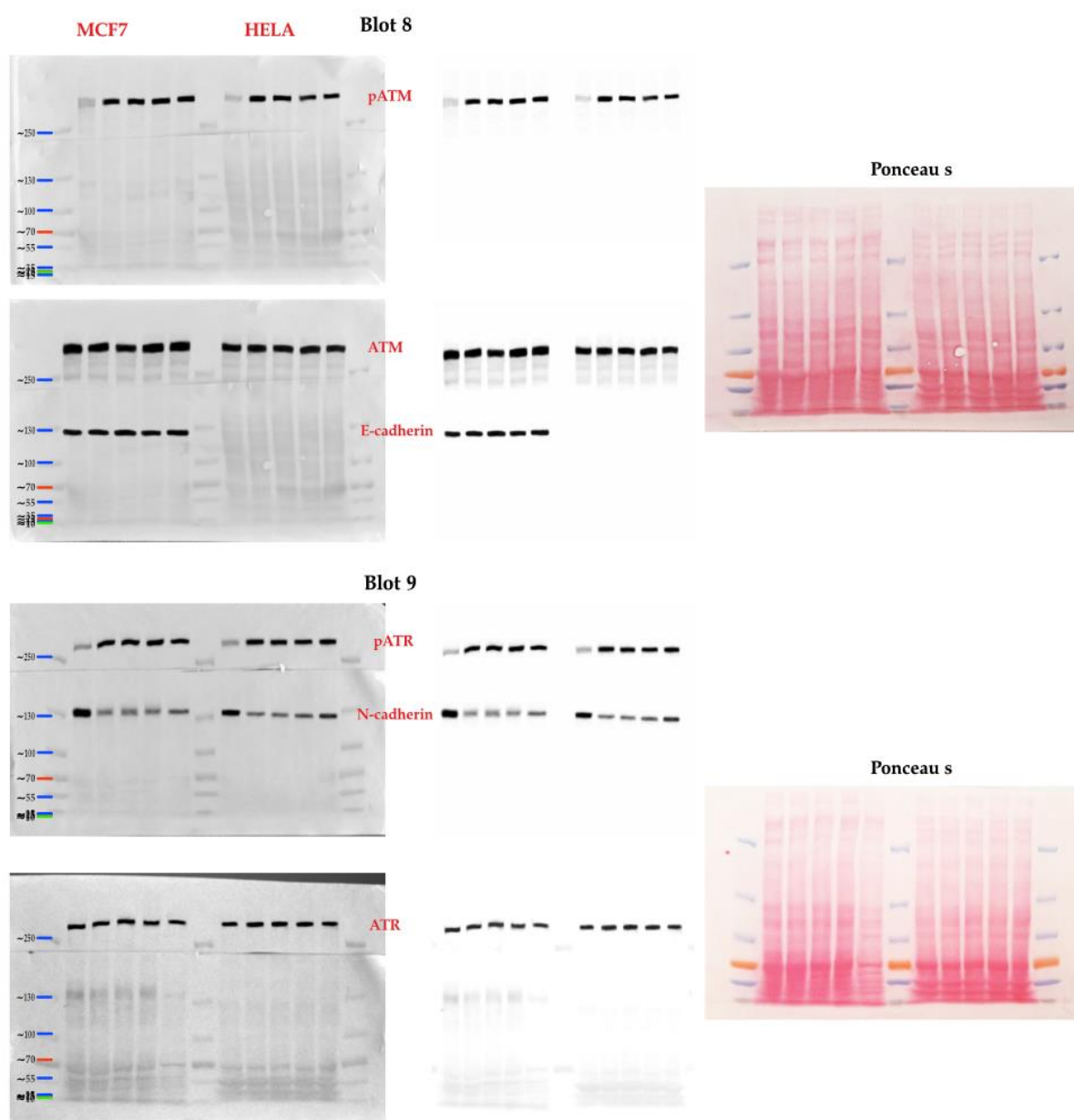
Figure 3. Raw data of western blot (full blots)

Figure 4. Distribution of differentially expressed genes across treatment groups.

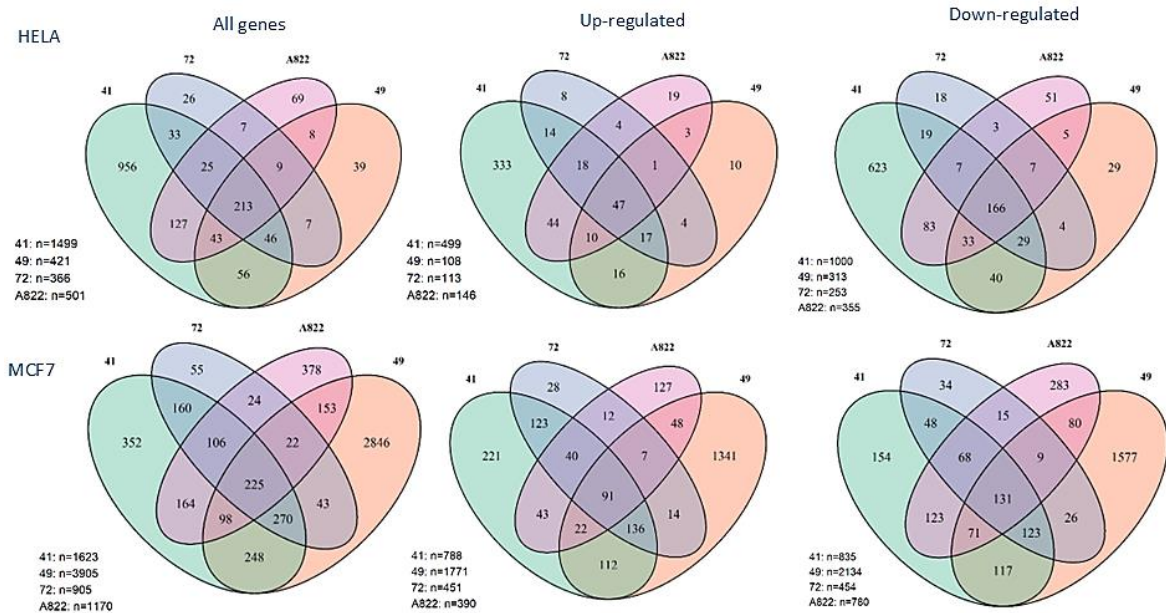
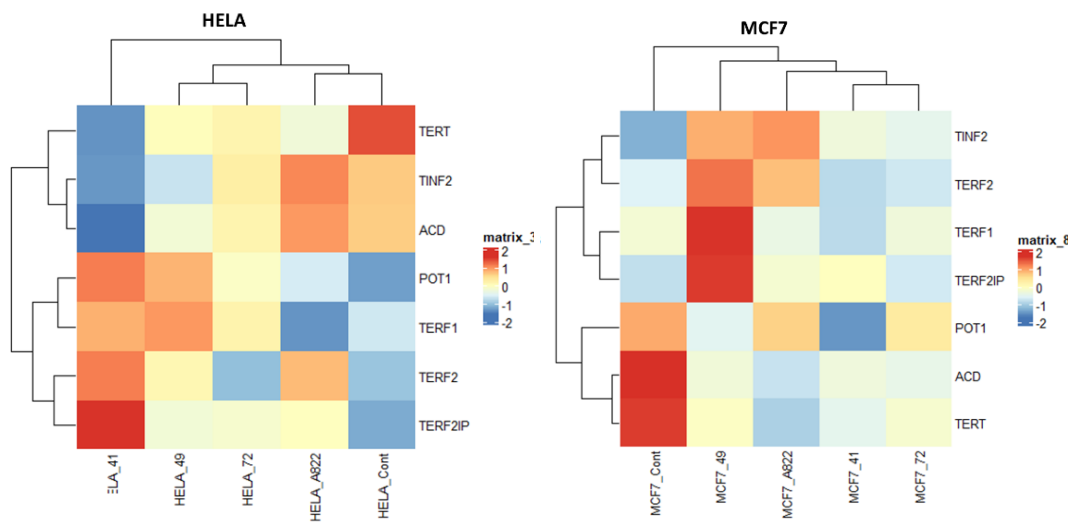


Figure 5. Distribution of mRNA expression levels of shelterin complex across treatment groups.



Scientific Achievements

Journal Publications

2026

Arya, C. G., Thomas, N. M., Chandrakanth, M., **Kallingal, A.**, Amrutha, P. G., Sivapriya, M. S., Gondru, R., & Banothu, J. (2026). Coumarin-benzimidazole hybrids: Design, synthesis and identification of potential anticancer agents. *Bioorganic & Medicinal Chemistry Letters*, 131, Article 130444. <https://doi.org/10.1016/j.bmcl.2025.130444> (IF: 2.3; Q2)

Golcienė, B., Maciejewska, N., **Kallingal, A.**, Sapijanskaitė-Banevič, B., Stasevych, M., & Mickevičius, V. (2026). Imidazole-2-thione derivatives as new selective anticancer agents with anti-metastatic properties: Synthesis and pharmacological evaluation. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 41(1), Article 2607820. <https://doi.org/10.1080/14756366.2025.2607820> (IF: 5.3; Q1)

Maciejewska, N., Ristić, P., **Kallingal, A.**, Gulea, M., Donnard, M., Girard, N., et al. (2026). Multifaceted biological and computational assessment of aromatic and N-heteroaromatic non-substituted thiosemicarbazones. *Scientific Reports*, 16, Article 14911. <https://doi.org/10.1038/s41598-026-44568-4> (IF: 4.9; Q1)

Thomas, N. M., **Kallingal, A.**, Maciejewska, N., Chandrakanth, M., Salunke, D. B., Petrovsky, N., Kundil, V. T., Abhithaj, J., Alharbi, M., Narajczyk, M., Gondru, R., Pasangha, C. H., & Banothu, J. (2026). Discovery of 1,2-dihydro-4-hydroxy-2-oxoquinoline-3-carboxamide derivatives as potent anticancer agents: Structure-based design, synthesis, mechanistic insights, target identification, and molecular docking studies. *Bioorganic Chemistry*, 175, Article 109783. <https://doi.org/10.1016/j.bioorg.2026.109783> (IF: 5.1; Q1)

2025

Fabitha, K., Chandrakanth, M., **Kallingal, A.**, Maciejewska, N., Telukuntla, P., Gondru, R., Kumavath, R., Demina, T. D., Alharbi, M., & Banothu, J. (2025). Identification of pyrazole-acrylonitrile derivatives as potential anticancer agents and mechanistic insights. *Bioorganic & Medicinal Chemistry*, 130, Article 118352. <https://doi.org/10.1016/j.bmc.2025.118352> (IF: 3.5; Q1)

Fabitha, K., **Kallingal, A.**, Maciejewska, N., Haneefa, H. H. P. T., Thachan Kundil, V., Alharbi, M., & Banothu, J. (2025). Improved anti-breast cancer activity through structural modification of fused pyran derivatives and mechanistic investigations. *RSC Medicinal Chemistry*, 16(8), 3671-3696. <https://doi.org/10.1039/D5MD00161G> (IF: 4.6; Q1)

Ristić, P. G., Maciejewska, N., **Kallingal, A.**, Rodić, M., Milivojac, A. L., Pregelj, M., et al. (2025). Structural patterns and cytotoxic activity of dinuclear Cu(II), Ni(II) and Cd(II) complexes with pyridine-based malonic acid dihydrazone. *Polyhedron*, 275, Article 117523. <https://doi.org/10.1016/j.poly.2025.117523> (IF: 2.8; Q2)

2024

Brankiewicz-Kopcińska, W., **Kallingal, A.**, Krzemieniecki, R., & Bagiński, M. (2024). Targeting shelterin proteins for cancer therapy. *Drug Discovery Today*, 29(8), Article 104056. <https://doi.org/10.1016/j.drudis.2024.104056> (IF: 8.7; Q1)

Fabitha, K., **Kallingal, A.**, Maciejewska, N., Arya, C. G., Chandrakanth, M., Thomas, N. M., Li, Y., Gondru, R., Munikumar, M., & Banothu, J. (2024). Novel fused pyran derivatives induce apoptosis and target cell cycle progression in anticancer efficacy against multiple cell lines. *New Journal of Chemistry*, 48(18), 8038-8054. <https://doi.org/10.1039/D4NJ00824C> (IF: 2.6; Q2)

Kallingal, A., Krzemieniecki, R., Maciejewska, N., Brankiewicz-Kopcińska, W., & Bagiński, M. (2024). TRF1 and TRF2: Pioneering targets in telomere-based cancer therapy. *Journal of Cancer Research and Clinical Oncology*, 150(7), Article 353. <https://doi.org/10.1007/s00432-024-05867-3> (IF: 3.3; Q1)

Olszewski, M., Maciejewska, N., **Kallingal, A.**, Chylewska, A., Dąbrowska, A. M., Biedulska, M., Makowski, M., Padrón, J. M., & Bagiński, M. (2024). Palindromic carbazole derivatives: Unveiling their antiproliferative effect via topoisomerase II catalytic inhibition and apoptosis induction. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 39(1), Article 2302920. <https://doi.org/10.1080/14756366.2024.2302920> (IF: 5.3; Q1)

Trocka, A., **Kallingal, A.**, Maciejewska, N., Narajczyk, M., Hromova, A., & Makowiec, S. (2024). Synthesis and biological evaluation of novel 3,6-amide and thioamide substituted-2,3,4,9-tetrahydro-1H-carbazoles for anti-cancer activity. *European Journal of Medicinal Chemistry*, 277, Article 116755. <https://doi.org/10.1016/j.ejmech.2024.116755> (IF: 6.7; Q1)

2023

Ayyolath, A., **Kallingal, A.**, Thachan Kundil, V., Suresh, A. M., & Jayadevi Variyar, E. (2023). Investigating the disease-modifying properties of sclerotiorin in Alzheimer's therapy using acetylcholinesterase inhibition. *Chemical Biology & Drug Design*, 102(2), 292-302. <https://doi.org/10.1111/cbdd.14244> (IF: 3.3; Q2)

Brankiewicz-Kopcińska, W., Kalathiya, U., Padariya, M., Węgrzyn, K., Prusinowski, M., Joanna Zebrowska J., Zylicz-Stachula A., Skowron P., Drab M., Szajewski M., Ciesielski M., Gawrońska M., **Kallingal A.**, Makowski M, and Bagiński M. (2023). Modified peptide molecules as potential modulators of shelterin protein functions; TRF1. *Chemistry-A European Journal*, 29(55), Article e202300970. <https://doi.org/10.1002/chem.202300970> (IF: 3.6; Q1)

Joseph, T. M., **Kallingal, A.**, Suresh, A. M., Mahapatra, D. K., Hasanin, M. S., Haponiuk, J., & Thomas, S. (2023). 3D printing of polylactic acid: Recent advances and opportunities. *The International Journal of Advanced Manufacturing Technology*, 125(3-4), 1015-1035. <https://doi.org/10.1007/s00170-022-10795-y> (IF: 3.7; Q1)

Kallingal, A., Olszewski, M., Maciejewska, N., Brankiewicz, W., & Bagiński, M. (2023). Cancer immune escape: The role of antigen presentation machinery. *Journal of Cancer Research and Clinical Oncology*, 149(10), 8131-8141. <https://doi.org/10.1007/s00432-023-04737-8> (IF: 3.3; Q1)

Note: Metrics can change after each annual Journal Citation Reports and Scopus/CiteScore release.

Patent applications

WIPO ST 10/C PL 453805, **Kallingal, A.**, Krzemieniecki, R., Hromova, A., Maciejewska, N., Bagiński, M., et al. *Chemical compounds with biological activity, especially anticancer, in particular modulating the functions of the telomeric protein TRF1, the method of obtaining them and the biological use of these compounds*. Filing date 20 November 2025

Conference poster presentations

Kallingal, A., Wegrzyn, K., Maciejewska, N., & Bagiński, M. *Targeting shelterin with small molecules induces telomere dysfunction: cellular and molecular consequences*. EMBO, Lazise, Italy, 25-30 May 2026.

Kallingal, A., Krzemieniecki, R., Maciejewska, N., & Bagiński, M. *Targeting the guardians of telomeres: A new approach for anticancer drug development* ENA EORTC, Barcelona, Spain, 26-28 October 2022

Krzemieniecki, R., **Kallingal, A.**, Maciejewska, N., & Bagiński, M. *Novel compounds for targeting protein-protein interactions in TRF1 and TRF2*. ENA EORTC, Barcelona, Spain, 26-28 October 2022

Maciejewska, N., **Kallingal, A.**, Narajczyk, M., Stasevych, M., Zvarych, V., & Bagiński, M. *Challenging immortality: Investigating the efficacy of novel telomerase inhibitors on telomere disruption and cancer elimination*. ENA EORTC, Barcelona, Spain, 26-28 October 2022

Trocka, A., **Kallingal, A.**, Maciejewska, N., Narajczyk, M., Hromova, A., & Makowiec, S. *Thioamide-functionalized tetrahydrocarazoles as potential anticancer agents*. 18th Copernican Doctoral Seminar, Gdansk, Poland, 26-27 June 2025

Conference-related publications in journal supplements and books of abstracts

Kallingal, A., Krzemieniecki, R., Maciejewska, N., & Bagiński, M. (2024). 64: Targeting the guardians of telomeres: A new approach for anticancer drug development. *European Journal of Cancer*, 211, Article 114591. <https://doi.org/10.1016/j.ejca.2024.114591> (IF: 7.9; Q1)

Krzemieniecki, R., **Kallingal, A.**, Maciejewska, N., & Bagiński, M. (2024). 371: Novel compounds for targeting protein-protein interactions in TRF1 and TRF2. *European Journal of Cancer*, 211, Article 114884. <https://doi.org/10.1016/j.ejca.2024.114884> (IF: 7.9; Q1)

Maciejewska, N., **Kallingal, A.**, Narajczyk, M., Stasevych, M., Zvarych, V., & Bagiński, M. (2024). 75: Challenging immortality: Investigating the efficacy of novel telomerase inhibitors on telomere disruption and cancer elimination. *European Journal of Cancer*, 211, Article 114602. <https://doi.org/10.1016/j.ejca.2024.114602> (IF: 7.9; Q1)



GDAŃSK UNIVERSITY
OF TECHNOLOGY

