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Targeting Shelterin Proteins For Cancer Therapy

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Abstract

Cancer, a global health challenge, prompts continuous exploration for innovative therapies also based on new targets. One promising avenue is targeting the shelterin protein complex, a safeguard for telomeres crucial in preventing DNA damage. Shelterin's role in modulating ATM and ATR kinases, key players in DNA damage response, establishes its significance. Disrupting shelterin's defence mechanisms especially in cancer cells makes telomeres vulnerable, potentially leading to genomic instability and hindering cancer cell survival. This review outlines recent approaches exploring shelterins as potential anticancer targets, highlighting the prospect of developing selective molecules to exploit telomere vulnerabilities toward new innovative cancer treatment.

Keywords: telomere, shelterin proteins, structure-based drug design, shelterin complex, shelterin protein's inhibitors,

Highlights/Teaser

- Shelterins are underexplored anticancer target

- Molecular structures of the shelterin’s protein complex are continuously delivered

- *In silico* technology can open new perspective in exploration shelterins as an anticancer target

Introduction

Cancer is a complex disease that arises from the uncontrolled growth and division of abnormal cells [1, 2]. Despite significant advancements in cancer diagnosis and treatment, it remains a major global health challenge. Therefore, there is a continuous effort to search for new therapies and new targets which can be utilised to fight with cancer. One new promising avenue for anticancer therapy is targeting the shelterin proteins complex, recently proposed as a new target [3–5].

The shelterin complex constitutes the so-called capping end of the chromosome telomeres, which is essential for their protection, preventing telomeres from fusion with other chromosome ends, reducing telomere fragility, and protecting them from degradation [6, 7]. The shelterin complex also plays a crucial role in preventing DNA damage at the telomeres. It allows DNA to form a lasso-like structure with a telomeric loop and a displacement loop that shields the 3′-end from DNA damage [8]. This shielding blocks the activation of DNA repair mechanisms such as ataxia-telangiectasia Rad3-related-mediated DNA damage kinase signalling and ataxia-telangiectasia mutation kinase cascades. ATM (ataxia-telangiectasia mutated) and ATR (Ataxia-Telangiectasia and Rad3-related) kinases function as sensors of DNA damage and play a crucial role in initiating and coordinating the cellular response to DNA damage [9]. Upon sensing DNA damage, ATM and ATR kinases are recruited to the site of damage, where they phosphorylate downstream targets involved in DNA repair and cell cycle arrest. ATM kinase primarily responds to DNA double strand breaks, while ATR kinase primarily responds to single-stranded DNA and stalled replication forks [10].

Additionally, the shelterin complex prevents unwanted repair reactions [11]. There are two main DNA damage repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) [12]. Non-homologous end joining is a pathway that repairs DNA double strand breaks by directly ligating the broken ends without the requirement for a homologous template. Homology-directed repair, on the other hand, utilizes a homologous template, typically a sister chromatid or a homologous chromosome, to repair DNA double strand breaks with high fidelity. The activation of ATM and ATR kinases in response to DNA damage leads to the recruitment and activation of various downstream proteins involved in these repair pathways. The shelterin complex, through its interactions with ATM and ATR kinases, regulates the activation of these DNA damage repair pathways [13]. The shelterin complex acts as a guardian for telomeres, preventing their recognition as DNA double strand breaks and activation of unnecessary DNA damage repair pathways such as non-homologous end joining and homology-directed repair pathways [7].

The vulnerability of defence mechanisms in telomeres has significant implications for targeting them as a potential anticancer strategy. With the loss of proper shelterin function, telomeres become susceptible to DNA damage and repair proteins. This compromise in protection can lead to genomic instability, increasing the risk of cancer development or its impairment, the latter one would be beneficial for cancer cell damage. Furthermore, targeting the integrity of telomeres and shelterin complex can disrupt telomere length maintenance, which is critical for cancer cell survival and proliferation [14]. By developing selective molecules that specifically target and disrupt shelterin proteins complex, it may be possible to exploit the compromised defence mechanisms in cancer cells telomeres and inhibit cancer growth. This approach could provide a novel therapeutic strategy for cancer treatment by exploiting the vulnerabilities of telomeres and their defence mechanisms. Our review covers different recent approaches which study shelterins as potential anticancer targets.

Shelterin Complex Architecture

One of the shelterin components are two homologous, homodimeric proteins which are called TRF1 (telomere-repeat binding factor 1) and TRF2 (telomere-repeat binding factor 2) [6]. Those proteins serve as a base or even platform for larger complexes formed by TIN2, TPP1, POT1 and RAP1 (as shown Figure 1).

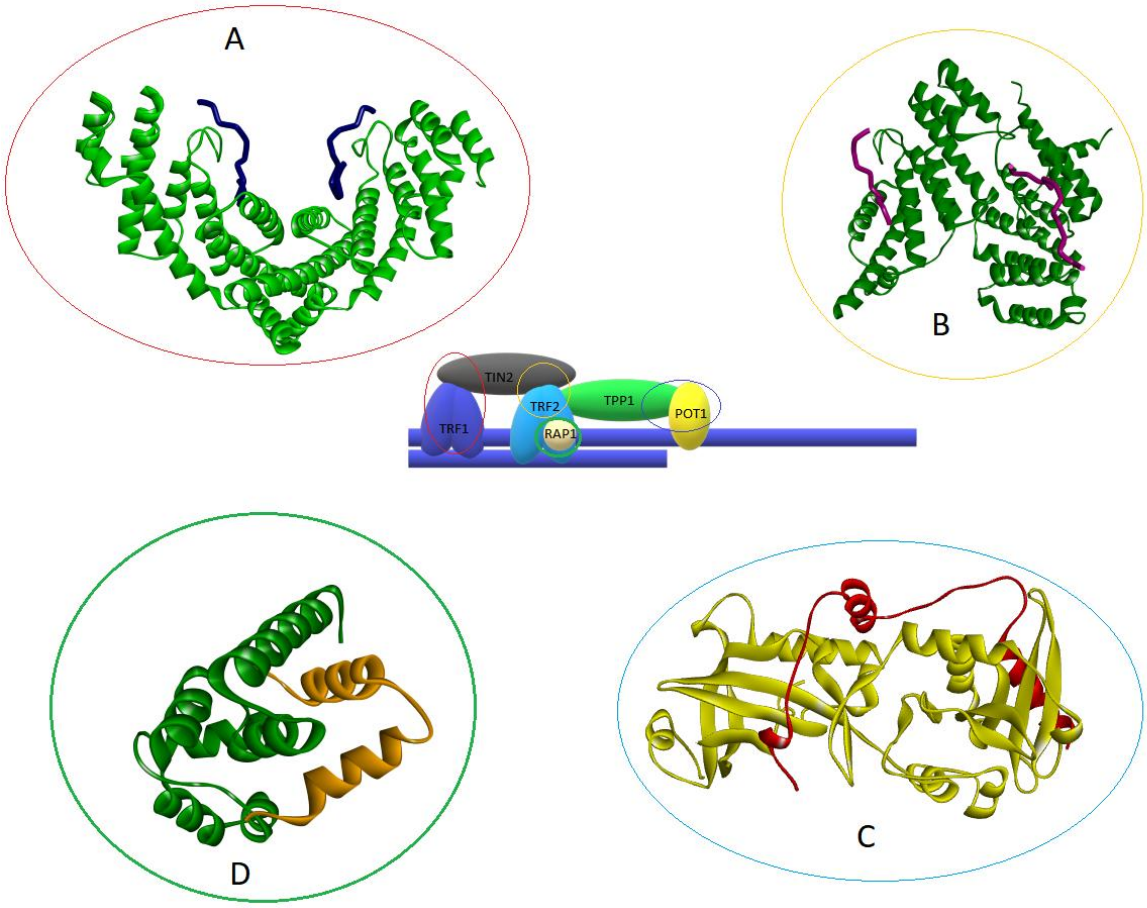

Figure 1. Graphical representation of the shelterin complex in the centre. A - Represents protein structure of TRF1 and TIN2. Light green is TRF1 dimer in solid ribbon representation, dark blue TIN2 in tubular representation. (PDB:3BQO); B - Shows a TRF2 and TIN2 complex structure. Dark green is TRF2 dimer in solid ribbon representation, magenta is TIN2 in tubular representation.(PDB:3BU8); C - Displays the TPP1 and POT1 interaction. Yellow is POT1 structure, red is TPP1 structure, both in solid ribbon are represented.(PDB:5H65),D - Represents an interface between TRF2 and RAP1. Green is RAP1 structure, orange is TRF2 structure both in solid ribbon are represented. (PDB:3K6G).

This is due to the fact that these proteins are the first line proteins binding DNA. TRF2 controls the topology of telomeric DNA to aid t-loop formation. To support the aforementioned function, RAP1 binds the TRF2. While TRF1 counteracts telomerase activity, the POT1-TPP1-TIN2 complex is essential for telomerase recruitment and activation. Hence, each component of the TRF1/2 sheltering protein plays its own role in telomerase maintenance. Their composition is generally compromised in to three main sections which are N-terminal domain - acidic in TRF1 (due to large number of glutamic acid [15]), basic in TRF2 (because of the fact that contains higher amounts of arginine [16]), central domain called TRF-homology (TRFH), responsible for the dimerization between the TRF1 or TRF2 monomers respectively, and C-terminal domain Myb, whose function is telomere recognition and binding

[17]. The role of amino acid sequence between central and Myb domain serves as a hinge in TRF1. Dimeric structures of both paralog forms in a reverse horseshoe shape and performed superposition shows little difference of their spatial conformation of TRFH [18]. Although high similarity of TRF1/2 telomere binding proteins, the heterodimerization amidst the homologs is not possible, due to the divergence in the dimer interface and large difference of buried surface area (TRF2 is by 247Å^2 wider than TRF1) [19]. Therefore, some important amino acids which are crucial for dimerization, exhibit distinct conformation and do not fit into the heterodimer.

Responsible for the DNA binding is a three-helical motif in a pattern of helix-turn-helix in human TRF1 and TRF2, located in C-terminal site, which is capable of recognition characteristic DNA telomeric sequence “TTAGGG” in 3’ chain. Both protein structures show similarity resulted in 70% [20]. Despite the fact of high similarity, the TRF1 binding domain is attaching stronger to DNA than its protein homology counterpart TRF2 [11]. For the identification of the proper DNA sequence by TRF1 in Myb domain the amino acids which possess positively charged side chain as Arg in position 425 and Lys421; negatively charged Asp422 and polar molecules Ser417, Met419 mostly are involved. These regions are responsible for recognition the major groove of telomeric TTAGGGTTAGGG framework. Furthermore, Arg 380 is responsible for recognition of minor groove site [17, 21]. TRF2 Myb-like domain is composed in the same manner as TRF1 DNA binding domain, the architecture of both are nearly the same, although certain changes in primary amino acid structure affect spatial organization of the binding domain of TRF2. In cognate TRF1, C-terminal Myb domain secondary structure consists of three alpha helices, which first (residues 19-31) is a β-turn IV stabilized by a two sets of salt bridges between a pair N-terminal Asn36 - C-terminal Gly35 and N-site Trp37 – C-site Gly35. Second α-helix differs from its TRF1 homologue, due to the presence of proline in position at residue 45, which induces an extra turn resulting in different spatial organization, but the third helix of TRF2 Myb domain preserves high similarity as its TRF1 counterpart. These three are connected with two turns, which are stabilized by non-covalent interaction, but also influence of specific amino acid as for example proline (resulting in specific ɣ turn) [22].

TRF - A versatile telomeric protein binder

TRF1 and TRF2 recruit several proteins, together they bind TIN2 (TERF-interacting nuclear factor 2), while for the TRF1 the responsible site for the binding is TRFH domain with $TIN2_{TBM}$ site (TRFH binding motif - TMB), in the paralog protein the interaction is taking place in $TRF2_{TBM}$ (TIN2 binding motif) [23]. TRF1, also binds with proteins outside the shelterin complex through the dimerization domain (Figure 2), those proteins must satisfy specific

amino acid sequence compromised with phenylalanine, leucine and proline separated by any other amino acid (FxLxP recognition motif x-represents any amino acid except Cys). This is fulfilled by PINX 1 and TERB1. The same role plays dimerization domain in TRF2, where concurrence of YxLxP motif of recognition is observed in proteins such as Apollo, NBS1 or SLX4 and interaction in TRF2 binding pocket was confirmed [24, 25]. Apollo is an Artemis-Related Nuclease, which interacts with TRF2 and protects human telomeres in S Phase [26].

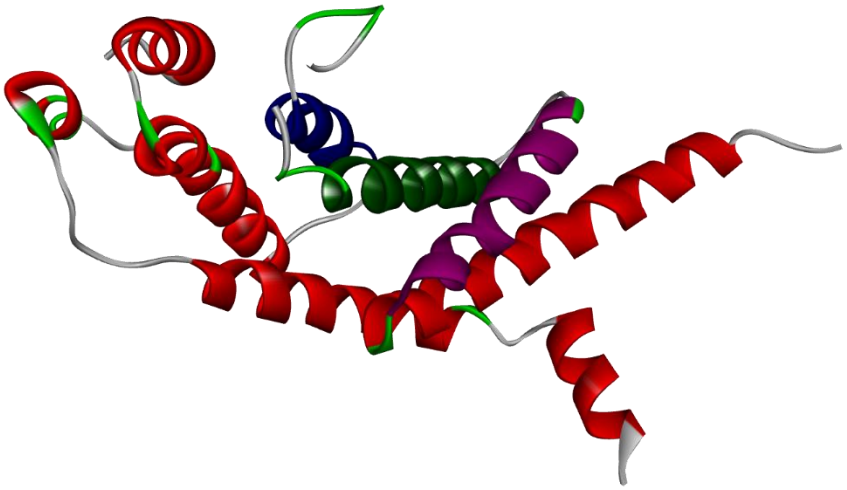

Figure 2. Monomer of dimerization domain of TRF1. In fuchsia is highlighted α2 chain, following green, which represents α3 helix lastly the dark blue short alpha helix, is α4. The distinguished by different colour chains are crucial for interaction with the TIN2 polypeptide.

Important sites in TRFH for binding TIN2

Most of the tertiary structure organization of TRF1 is composed by alpha helices, in total count of twenty, ten per each monomer. Through those helixes, the $TIN2_{TBM}$ interacts with α2, α3 and α4 also with unstructured loop34, the interaction is stabilized mostly by hydrogen bond interactions and important stacking-pi interaction, between Phe142 of TRF1 and Pro262, which is important in protein recognition of binding, it compromises with binding affinity of 3.14 mM [15, 27]. TRF2 contains two sites in order to make a stable connection with TIN2, one of them is the TRFH domain, which also has ten helixes and another site is short protein sequence of $TRF2_{TBM}$ in 352-365 position of amino acids. Surprisingly, the key role for interaction between TRF2 and TIN2 plays the short amino acid of TBM region, rather than the TRFH domain of this protein. The reason lies in exhibited different roles between those two paralogs, each of those two proteins interacts with different associated functional polypeptides, therefore key amino acids are changed, displaying different electrostatic

potential distribution and changed shape of the binding pocket for their counter partners [22–24].

TRF2-Rap1 – recruitment of Rap1 protein.

Another shelterin component which associates with TRF platforms is Rap1 (Repressor/Activator 1). This polypeptide also exhibits six α-helices motif, which interacts with TRF2, where the major role plays weak forces - Van der Waals interaction together with hydrophobic interactions. Rap 1 RCT domain (Rap C-Terminal) with α1 and α2 interacts with α1 and α2 $TRF2_{RBM}$ (RAP1 Binding-Motif), where one of the most important roles plays Leu in position 288, where makes an important hydrophobic interaction, fitting in the pocket of α1 of Rap1 [28]. Research of potential polypeptide inhibitors, measuring binding affinity for each amino acid substitution, showed that Leu 295 is crucial. The $TRF2_{RBM}$ structure consists of three alpha helices and four unorganized polypeptides [29].

POT1-DNA interaction

While the TRF1 and the TRF2 are specialized structures for binding double-stranded DNA, the POT1 (Protection of Telomere 1) is responsible for recognition and attachment of single-stranded DNA so called 3'-overhang [30]. Although only POT1 can form a complex with DNA, the studies provided by Wang F, et al. showed that TPP1 (Telomere Protection Protein 1) provides higher stability of the protein POT1-DNA structure [31].

TPP1-POT1 structure and interaction

The TPP1 domain consists of 66 amino acids, which are responsible for binding with the POT1 structure. The dominating interactions are hydrophobic interactions, where, for example, the Leu271 of TPP1 helix α1 is buried in a hydrophobic pocket of POT1 surface. The TPP1 helix α2, makes interaction between the OB-fold and HJR domain, and the parallel case as Leu271, the Trp293 is buried in a hydrophobic pocket of the OB fold. The last two helices of TPP1 binding domain are the α3 and α4, which are responsible for additional interactions with the POT1 OB fold, it is worth to mention that Tyr306 of TPP1 stacks with the Pro371 of POT1 and the Ile315 serves as a linker between the α3-α4 chains and the POT1. Lastly, the last chain of α4 shows limited interactions with the POT1C, where an example of interaction may be between side chain of Leu325 of TPP1 and Pro357 with Lys608 of POT1 [32].

The Role of the Shelterin Complex in Non-cancer and Cancer Cells

Non-Cancer Cells

The role of the shelterin complex in non-cancer cells is multifaceted and essential for maintaining genomic stability. Beyond its direct involvement in protecting telomeres from DNA damage response pathways, the intricate interplay of its constituent proteins underscores the complexity of telomere regulation. Specifically, the synergistic action of TRF1 and TRF2 in binding to double-stranded telomeric DNA repeats and the interaction of POT1 with single-stranded telomeric DNA highlights the meticulous mechanisms by which the shelterin complex safeguards telomeres [33]. Moreover, the control of telomere replication through the regulation of telomerase access showcases the intricate molecular orchestration required for the maintenance of telomeric integrity [34, 35]. The prevention of chromosome end fusion by the shelterin complex is a pivotal aspect of its function, as it safeguards against genomic instability and the potential dysregulation of cellular processes [36]. This regulatory framework underscores the critical role of the shelterin complex in ensuring the stability and functionality of non-cancer cells. Furthermore, the shelterin complex has been implicated in playing a role in cellular senescence and aging. Recent research in the field has further elucidated the role of the shelterin complex in non-cancer cells, shedding light on its involvement in cellular senescence and aging. Studies have demonstrated that the shelterin complex not only protects telomeres from DNA damage but also participates in orchestrating the cellular response to replicative stress, ultimately impacting cellular senescence [37]. This expanded understanding underscores the multifaceted nature of the shelterin complex and its far-reaching implications in cellular physiology. Moreover, the intricate interplay of the shelterin complex's constituent proteins extends beyond telomere protection to encompass broader aspects of genomic stability [38]. Research has highlighted the potential implications of the shelterin complex in maintaining not only telomeric integrity but also overall genome stability, suggesting a more holistic role in preserving cellular homeostasis [39]. Furthermore, the intricate regulatory mechanisms employed by the shelterin complex underscore the complexity of telomere regulation and its impact on cellular function. The detailed interplay between TRF1, TRF2, POT1, and other associated proteins exemplifies the meticulous orchestration required for safeguarding telomeres and maintaining genomic stability. Overall, the evolving understanding of the Shelterin complex in non-cancer cells underscores its essential role in cellular physiology, genomic stability, and aging, painting a more comprehensive picture of its significance in cellular function and maintenance.

Cancer cells

The shelterin complex, a crucial regulator of telomere function, has emerged as a focal point in cancer research, with multiple studies revealing its intricate involvement in various types of cancer. In breast cancer, the downregulation of TRF1 has been consistently reported, linked

to the overexpression of miR-155, an oncomiR targeting TRF1's 3′UTR [40]. This downregulation correlates with longer telomeres in cancer cells, facilitating prolonged proliferation. Additionally, TIN2 upregulation in breast cancer cell lines suggests its role in promoting cell proliferation and migration [41]. Conversely, TRF2 is upregulated in breast cancer, safeguarding critically short telomeres from DNA damage recognition, thereby preventing apoptosis [42]. RAP1 and NF-κB levels are highly correlated, contributing to higher breast cancer grades [43]. In lung cancer, TRF1 and TRF2 expression increases with disease progression, providing tolerance to short telomeres and preventing apoptosis [44]. Colorectal cancer patients display disrupted telomeric homeostasis, with telomeric length inversely correlated with DDR pathway activation [45]. Prostate cancer exhibits upregulation of TRF1 and TIN2, while gastric cancer showcases diverse alterations in shelterin components, influencing telomerase activity, telomere length, and cell immortalization [46]. Hepatocellular carcinoma demonstrates progressive upregulation of TRF1, TRF2, TIN2, and POT1, contributing to telomere shortening and increased chromosomal instability [47]. Glioblastoma displays varying expression patterns of TRF1, TRF2, and POT1, influencing telomere shortening, chromosomal instability, and prognosis [48]. Leukaemias, including acute lymphocytic leukaemia and adult T-cell leukaemia, exhibit upregulated shelterin components, leading to telomere shortening and increased genetic instability [49]. Chronic lymphocytic leukaemia displays complex alterations, with upregulation of TRF1, RAP1, and TPP1 [50], while chronic myeloid leukaemia exhibits initial upregulation followed by downregulation of TRF1 [51] and TRF2 [49]. Non-small cell lung cancer experiences upregulation of TRF1 and TRF2, contributing to telomere dysfunction and altered checkpoint controls [52]. Pancreatic cancer [53], renal cell carcinoma [54], head and neck squamous cell carcinoma [55], classical Hodgkin lymphoma [56], skin cancer [57], familial papillary thyroid cancer [58], splenic marginal zone lymphomas [59], and melanoma [60] all demonstrate unique alterations in shelterin components, influencing various aspects of tumorigenesis. In summary, mutations in shelterin complex genes are evident across diverse cancers, emphasizing their potential role in tumorigenesis. While these mutations are relatively rare, the intricate involvement of the shelterin complex in telomere maintenance and genomic stability underscores its significance in cancer development. Further research is crucial to unravel the precise mechanisms and therapeutic potential of targeting the shelterin complex in specific cancer types.

Shelterin Proteins as an Emerging Anti-Cancer Target

Since different levels and mutations of telomeric proteins are observed in cancer cells and their role for maintaining chromosome stability in the latter cells seems to be more important than in normal cells due to usually shorter telomeres in malignant cells, targeting shelterins looks promising [3, 4, 61]. Different approaches have been already applied to target shelterin complex or its components alone by small molecules or peptide-like structures and different biological effect have been recorded (Table 1). In general strategies to target shelterin complex may cover blocking access of TRF1 or TRF2 as well as POT1 to the DNA. Another approach may be responsible for regulation of expression levels of key protein components of shelterin complex. The third type of action is inhibition of binary interactions between shelterin components. The first approach is more general and is mostly focus on stabilisation of non-canonical 2D DNA structures (usually G-quadruplex structures) which can block access of different proteins to DNA [62, 63]. However, this interaction of different ligands with telomeric DNA is not very selective because telomeric G-rich sequences has been discovered also in many promoter regions of genes [64]. Thus this approach does not guarantee selective blocking access of shelterin proteins to DNA. Taking into account this fact two other approaches look more promising. However, modulation of expression of shelterin proteins or its degradation is also challenging since it may target also normal cells function. The experiments with silencing particular genes or degradation of shelterins also showed that lack of certain proteins is lethal for cells [65, 66]. Concerning this fact, one may say that the most promising and still not very extensively explored approach should be inhibition or modulation of the interaction between components of shelterin complex. This approach is also very challenging because it leads to inhibition of protein-protein interactions. Such inhibition is not easy since usually requires spatial molecules, peptides or peptidomimetics. Two strategies can be applied to find or design such protein-protein inhibitors. One is High-Throughput Screening (HTS) which can be used for identifying chemical compounds that can modulate shelterin function. HTS enables the rapid testing of a large number of chemical substances for activity in diverse areas of biology [67]. Currently, HTS has become the most common approach for identifying starting points for drug development, with a growing number of publicly accessible HTS databases allowing researchers access to a large volume of HTS results [68]. The second approach is *in silico* technology to find or to design new molecules modulating protein-protein interactions in shelterin complex. *In silico* methods involve different computational methods to predict potential inhibitors or modulators of proteins. This concept of drug discovery is based on the computational procedure named structure-based drug design. In order to use this approach molecular structures of different shelterin proteins and their binary complexes should be

known and subsequently use for drug search. Therefore, a lot of efforts have been dedicated to define protein structures of shelterins and many of these structures have been already resolved (Figure 1 and Figure 2) [69, 70].

Nevertheless, many structures have been resolved the most explored structures toward drug finding is complex TRF1/TRF2-TIN2 [71–73] and TRF2-Apollo [74, 75] as well as TRF2-Rap1 [29]. Concerning structure-based drug design approach two strategies can be applied, one is Virtual High-Throughput Screening (VHTS) or the second *de novo* drug design. Since shelterin components are proteins, therefore to inhibit their mutual interactions the studies have been conducted mostly on modified peptide molecules as potential modulators of the protein functions. Particularly the TRF1 and TRF2 proteins demonstrate the application of *in silico* designed "peptidomimetic" molecules as potential modulators of their interaction with other components of shelterins. Modified peptides or peptidomimetics have been mostly designed using *de novo* design approach. Peptides either with single modified residue or extensively modified structures were design to mimic TIN2 or Apollo protein interacting with TRFs. There were also efforts to design stapled peptides targeting Rap1-TRF2 protein-protein interaction in shelterin complex. Concerning efforts to design or find small molecules to block protein-protein interaction within shelterins also TRF1 or TRF2 proteins were used. In this case HTS technology was applied and different molecules have been selected [72, 72, 73]. Thus targeting shelterin components using modified peptides or small molecules has emerged as a promising approach for cancer therapy.

Altogether*, in vitro* and *in vivo* experiments have been instrumental in elucidating the role of the shelterin complex in cancer and in evaluating the potential of drugs targeting this complex. Moreover, some *in silico* approaches have started recently and also delivered new molecules. The summery of different approaches and discovered or designed molecules is presented in Table 1.

Table 1. The list of shelterin complex inhibitors together with the short description of their mechanism of action and the way of their discovery:

Compound	Mechanism	Ref.
6-ThiodG (6-thio-2'-deoxyguanosine) Identified by *in vitro* screening	A nucleoside analogue that, once incorporated into telomeric DNA, modifies the structure of telomeres, thereby inhibiting the binding of TRF2.	[76]
A822 and B327 Identified by *in silico* HTS	Small molecules blocking (*in vitro* confirmed) interactions between TIN2 and TRF1/TRF2, respectively and exhibiting anticancer activity.	[77]
Alexidine·2HCl Identified by *in vitro*	Alexidine·2HCl, was found to impede tumour growth, neo-angiogenesis, and immunosuppression by downregulating TRF2 expression	[78]

screening		
Alisertib (ETP-51634) Identified by HTS screening in cells.	Treatment with ETP-51634 leads to telomere shortening and reduced TRF1 expression in lung cancer cells, indicating that the drug may have a significant impact on telomere maintenance and TRF1 function. ETP-51634-induced DNA damage activates the ATM kinase pathway, which subsequently phosphorylates and destabilizes TRF1, ultimately leading to telomere dysfunction.	[79]
APOD (Apolipoprotein D) Identified by *in vitro* and *in silco* screening and validation	APOD interacts with the TRF2 protein and promotes the formation of the T-loop structure at telomeres, which is essential for telomere protection and stability. APOD regulate telomere length and contribute to cellular senescence through its interaction with TRF2.	[71]
APOD53 cyclic peptide Identified by *in vitro* and *in silco* screening and validation	APOD53 forms a covalent adduct with a reactive cysteine residue present in the $TRF2_{TRFH}$ domain and induces phenotypes consistent with $TRF2_{TRFH}$ domain mutants. These include induction of a telomeric DNA damage response, increased telomeric replication stress, and impaired recruitment of RTEL1 and SLX4 to telomeres.	[80]
AR-A014418 Identified by *in vitro* screening and validation	AR-A014418 impaired tumour growth, neo-angiogenesis and immunosuppression by downregulating TRF2.	[78]
Arsenic trioxide (As_2O_3) Identified by *in vitro* screening and validation	Treatment with As_2O_3 led to a decrease in TRF2 protein levels and telomere dysfunction.	[81]
Berberine (Sysu-00692) Identified by *in vitro* screening and validation	Berberine demonstrates the ability to interfere with the binding between human POT1 and telomeric DNA. The compound exhibits mild inhibitory effects on telomerase activity and cell proliferation.	[82]
Congo red (CR) Identified by *in vitro* and also proved by *in silco*	CR can disrupt the interaction between POT1 *in vitro.*	[83]
Curcusone C Identified by HTS in cells	Curcusone C binds to the TRF2 protein and blocks its localization in DNA, which induces DNA damage response (DDR) and ultimately cell death in cancer cells.	[84]
Dasatinib (ETP-51801) Identified by *in vitro*	The treatment with ETP-51801 led to telomere shortening and reduced TRF1 expression in chronic myeloid leukaemia cells. Dasatinib-induced DNA damage led to activation of	[85]

	the ATM kinase pathway, which in turn phosphorylated and destabilized TRF1, leading to telomere dysfunction.	
Epigallocatechin-3-gallate (EGCG) Identified by *in vitro*	EGCG inhibits the binding of TRF1 to telomeric DNA.	[86]
ETP-47228 Identified by HTS in cells	The chemical compound identified to reduce TRF1 and it is not mentioned specifically which way it will work.	[72]
ETP-47037 Identified by HTS in cells	The chemical compound identified to reduce TRF1 and it is not mentioned specifically which way it will work.	[72]
ETP-50946 Identified by HTS in cells	The chemical compound identified to reduce TRF1 and it is not mentioned specifically which way it will work.	[72]
FKB04 Identified by *in vitro*	Discovery of a selective TRF2 inhibitor FKB04 induced telomere shortening and senescence in liver cancer cells, inhibition of TRF1 expression.	[87]
Flavopiridol (ETP-47306) Identified by HTS in cells	ETP-47306 interacts with components of the shelterin complex, including TRF1 and TRF2 (though the specific molecular mechanism is not yet known), indicating its involvement in the regulation of telomere maintenance and function.	[79]
Geldanamycin (ETP-50853) Identified by HTS in cells	ETP-50853 downregulates TRF1 expression and induce telomere dysfunction and apoptosis in cancer cells.	[79, 85]
Gemcitabine (ETP-45337) Identified by HTS screening in cells	Treatment with ETP-45337 led to telomere shortening and reduced TRF1 expression in pancreatic cancer cells. ETP-45337-induced DNA damage led to activation of the ATR kinase pathway, which in turn phosphorylated and destabilized TRF1, leading to telomere dysfunction.	[79, 85]
GSK461364 (ETP-51799) Identified by HTS screening in cells	ETP-51799 interacts with several components of the telomere-associated shelterin complex, including TRF1 and TRF2 (though the precise molecular mechanism is not known), and has been implicated in the regulation of telomere length and stability.	[79, 85]
KU-0063794 (ETP-50537) Identified by HTS screening in cells	ETP-50537 interacts with several components of the telomere-associated shelterin complex, including TRF1 and TRF2 (though the precise molecular mechanism is not known), and has been implicated in the regulation of telomere length and stability.	[79, 85]
MiR-182-3p Identified by *in silico* and by *in vitro* screening and validation	MiR-182-3p targets TRF2 and impairs tumour growth of triple-negative breast cancer.	[88]
MST-312 (3,6-bis(1-methyl-4-vinylpyridinium)carbazol	The peptide specifically targets the TRF1 and TRF2 protein and inhibits its interaction with telomeric DNA, leading to telomere dysfunction and eventual	[89]

e diiodide) Identified by *in vitro* screening and validation	cell death.	
PEP1 Identified by *in vitro* and *in silco* screening and validation	The peptide that mimics the core of the TIN2 binding domain. The molecule interferes with the binding of TIN2 to TRF1 *in vitro*.	[73]
RHPS4 (3,11-Difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate) Identified by *in vitro* screening and validation	A small molecule that binds to the G-quadruplex structure formed by the telomeric DNA and inhibits the binding of TRF2 to the telomeres.	[90–92]
SCH772984 (ETP-50728) Identified by HTS screening in cells	A small molecule inhibitor of the extracellular signal-regulated kinase (ERK) pathway. The ERK pathway has been shown to interact with several components of the shelterin complex, including TRF1 and TRF2 (not known molecular mechanism), and has been implicated in the regulation of telomere maintenance and function.	[79, 85]
Selumetinib (ETP-51667) Identified by HTS screening in cells	A small molecule inhibitor of the mitogen-activated protein kinase kinase (MEK) pathway. The MEK pathway has been shown to interact with several components of the shelterin complex, including TRF1 and TRF2 (not known molecular mechanism), and has been implicated in the regulation of telomere maintenance and function.	[79, 85]
Sirtinol Identified by HTS screening in cells	A small molecule inhibitor of the sirtuin family of NAD+-dependent deacetylases, which regulates TRF2 activity.	[93]
ZINC00005600 (Acacetin) Identified by *in silico* screening and validation	Interactions with the POT1 protein of these compounds possibly interrupt the natural state binding of POT1 with telomeric ssDNA, thus probably enhancing the telomere uncapping, elongation of the telomere, and chromosomal aberration which finally leads to cell death.	[94]
ZNC00020258 (3,3'-Methylenebis(2-hydroxy-4H-chromen-4-one) Identified by *in silico* screening and validation		

However it is worth to note, that these findings underscore the importance of *in vitro* experiments in elucidating the mechanisms underlying the role of the shelterin complex in cancer and in identifying potential therapeutic targets. The *in vivo* experiments also have provided valuable insights into the therapeutic potential of drugs targeting the shelterin

complex [61]. For example, the identification of shelterin complex-related signatures in oral squamous cell carcinoma has been proposed as a novel prognostic marker for cancer and a potential target for tumour therapy [95]. Additionally, the role of miR-185 in inducing telomere dysfunction and cellular senescence highlights the potential of microRNA-based interventions targeting shelterin components for cancer treatment [96]. The development of drugs targeting the shelterin complex in cancer has also been supported by the exploration of hybrid drugs that simultaneously target multiple points of signalling networks and various structures within cancer cells. The synergistic effects of complex drug combinations, such as dual inhibition of MAPK-ERK pathway components and combined inhibition of MEK and components of the PI3K signalling pathway, have shown promise in *in vitro* and clinical cancer research [97, 96]. To this end all approaches synergistically utilise knowledge from *in silico*, *in vitro* and *in vivo* studies and thus hopefully will bring new drug candidate molecules.

Conclusions and future directions

It is worth to note that after telomerase and telomeric DNA shelterin proteins became the centre of interests. The shelterin complex has garnered substantial attention in basic and clinical research, with a primary focus on its implications in cancer and aging-related diseases. Aberrations in shelterin complex genes have been meticulously documented across various cancer types, presenting a promising avenue for diagnostic and prognostic applications and for search of new anticancer targets. These genetic alterations not only serve as distinctive markers for identifying specific cancers but also offer insights into predicting the likely course of the disease, contributing to more informed and personalized patient care, especially if all these abnormalities will be understood at molecular level.

As our understanding of the shelterin complex deepens, the prospect of targeting its components emerges as a novel and promising therapeutic strategy for cancer treatment. The intricate interplay between shelterin proteins and telomeric DNA has been unravelled, revealing their multifaceted roles in the initiation and progression of various cancers. This newfound knowledge not only enhances our grasp of the underlying molecular mechanisms driving cancer but also points towards the development of targeted therapies that could disrupt these specific pathways. Such precision medicine approaches hold the potential for a more effective and tailored treatment strategy, minimizing adverse effects and optimizing outcomes. The clinical implications of shelterin complex aberrations extend beyond cancer to encompass also aging-related diseases. Research into the biological processes influenced by shelterin complex dysfunction has sparked a new wave of exploration into age-related pathologies. Unravelling the molecular intricacies of how the shelterin complex contributes to

aging opens up avenues for innovative approaches to managing and intervening in age-related diseases. This knowledge could potentially lead to therapies addressing the root causes of age-related pathologies, presenting a paradigm shift in the way these conditions are approached and treated. Furthermore, shelterin complex components are increasingly recognized as promising targets for precision medicine. The ability to specifically target the shelterin complex opens avenues for personalized treatment strategies, revolutionizing cancer treatment and providing targeted solutions for aging-related conditions. This personalized approach holds the potential to improve treatment outcomes, minimize side effects, and enhance the overall quality of life for patients.

In conclusion, the development of shelterin protein inhibitors or modulators encounters several challenges. One major challenge in developing shelterin inhibitors for cancer therapy is to target cancer cells only and spare normal cells. Since telomeres and shelterin complexes are also present in normal cells, it is important to develop strategies to selectively target cancer cells while minimizing toxicity to normal cells or to elaborate inhibitors which action based on the same target will be more harmful for cancer cells than normal. This goal is very challenging but using detailed structures of all components of shelterin complex and applying advanced in silico methods as well as extensive *in vitro* tests it will be possible to introduce more selective therapies. Another challenge is to identify which specific types of cancer would be eradicated the most from these therapies.

Overall, the future of shelterin inhibitors in cancer therapy looks promising, but more research is needed to fully understand their potential for cancer treatments and to develop strategies leading to minimization of potential side effects. This is still developing and underexplored area of medicinal chemistry where structural biology and in silico methods will play pivotal role.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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