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Modified Peptide Molecules As Potential Modulators of Shelterin Protein Functions; TRF1

Wioletta Brankiewicz,[a] Umesh Kalathiya,[b] Monikaben Padariya,[b] Katarzyna Węgrzyn,[c] Maciej Prusinowski,[d] Joanna Zebrowska,[d] Agnieszka Zylicz-Stachula,[d] Piotr Skowron,[d] Marek Drab,[e] Mariusz Szajewski,[f, g] Maciej Ciesielski,[f, g] Małgorzata Gawrońska,[d] Anoop Kallingal,[a] Mariusz Makowski,*[d] and Maciej Bagiński*[a]

In this work, we present studies on relatively new and still not well-explored potential anticancer targets which are shelterin proteins, in particular the TRF1 protein can be blocked by in silico designed "peptidomimetic" molecules. TRF1 interacts directly with the TIN2 protein, and this protein-protein interaction is crucial for the proper functioning of telomere, which could be blocked by our novel modified peptide molecules. Our chemotherapeutic approach is based on assumption that modulation of TRF1-TIN2 interaction may be more harmful for cancer cells as cancer telomeres are more fragile than in normal cells. We have shown in vitro within SPR experiments that our modified peptide PEP1 molecule interacts with TRF1, presumably at the site originally occupied by the TIN2 protein. Disturbance of the shelterin complex by studied molecule may not in short term lead to cytotoxic effects, however blocking TRF1-TIN2 resulted in cellular senescence in cellular breast cancer lines used as a cancer model. Thus, our compounds appeared useful as starting model compounds for precise blockage of TRF proteins.

Introduction

Short peptides with anticancer activity belong to two distinct categories: (i) natural peptides exhibiting sometimes also antibacterial activity, which bind to cancer cells exploiting evolution-acquired specificities, and (ii) designed by in silico prediction and chemically synthesized peptides, with precision molecular targeting of cancer cells, mostly specific proteins.[1,2] Unfortunately, naturally occurring peptides usually possess hemolytic activity that often prevents their use in in vivo therapy. Moreover, longer peptides are limited by less efficient transportation properties. Therefore, efforts are aimed towards generation of stable synthetic short peptides or modified peptides, that exhibit required properties yet lack undesired off-target effects. Properly designed, subsequently synthesized and in vitro validated peptides ensure desired molecular targeting pathogenic cells subpopulation in cancer patients (personalized targeting).[3]

In anticancer chemotherapy, many cellular targets have been used so far.[4–7] However, recently shelterin proteins have been proposed as new-class targets for anticancer strategy.[8–11] The studies of these targets are still very new and it is an unexplored area but is promising and relevant.[12–15] Thus targeting shelterin components by short peptides/modified peptides or small molecules emerge as a new concept for cancer therapy.[15–19] Telomeres (chromosome's ends), nucleo-

[a] W. Brankiewicz, A. Kallingal, M. Bagiński
Department of Pharmaceutical Technology and Biochemistry
Faculty of Chemistry
Gdansk University of Technology
Narutowicza St 11/12, 80-233 Gdansk (Poland)
E-mail: maciej.baginski@pg.edu.pl

[b] U. Kalathiya, M. Padariya
International Centre for Cancer Vaccine Science
University of Gdansk
ul. Kładki 24, 80-822 Gdańsk (Poland)

[c] K. Węgrzyn
Intercollegiate Faculty of Biotechnology
University of Gdansk
Abrahama 58, 80-307 Gdansk (Poland)

[d] M. Prusinowski, J. Zebrowska, A. Zylicz-Stachula, P. Skowron, M. Gawrońska, M. Makowski
Faculty of Chemistry
University of Gdansk
Wita Stwosza 63, 80-308, Gdansk (Poland)
E-mail: mariusz.makowski@ug.edu.pl

[e] M. Drab
Unit of Nanostructural Bio-Interactions
Hirszfeld Institute of Immunology and Experimental Therapy
Polish Academy of Sciences
12 Weigla-Street, 53-114 Wrocław (Poland)

[f] M. Szajewski, M. Ciesielski
Department of Oncological Surgery
Gdynia Oncology Centre
Gdynia (Poland)

[g] M. Szajewski, M. Ciesielski
Division of Propaedeutics of Oncology
Medical University of Gdańsk
Gdańsk (Poland)

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protein structures at the end of chromosomes stabilized by shelterin complex, in cancer cells often escape normal shortening and the tumor cells become quasi-immortalized and dividing beyond Hayflick limit, gaining survival advantage over normal cells.[20] Combating this cancers' survival strategy by interfering with the shelterin complex promises an attractive alternative to other anti-cancer strategies.[21]

Shelterin complex is composed of six proteins: telomeric repeat binding factor 1 (TRF1), and 2 (TRF2), repressor/activator protein (RAP1), protection of telomeres protein (POT1), TRF1-interacting nuclear protein 2 (TIN2), and TIN2- and POT1-interacting protein (TPP1).[20,21] This shelterin complex binds specifically to telomeric DNA.[20,22,23] These capping structures have the crucial function of assuring genome stability by protecting the chromosome end from being recognized as DNA double-strand breaks (DSBs).[22] Telomeres shelterin complex also represent challenging structures for the replication machinery, which is associated with telomere fragile sites.[24–26]

TRF1 is a key member of the shelterin complex. TRF1 and TRF2 proteins directly bind DNA TTAGGG telomere repeats and recruit the remaining shelterin proteins therefore TRFs are critical determinants of telomere's protection[20,27] TRF1 comprising 439 amino acids possesses a specific conserved domain (TRFH) which assists in the formation of a stable homodimeric TRF1-TRF1 structure. TRF1's myb-domains (two per a homodimer) enable dimer's stable interaction with the duplex DNA at the telomere. TRF1 plays a key role in the assembly of the shelterin complex by recruiting/binding to Telomere Repeat Binding Factor2 (TRF2) via TRF1 Interacting Nuclear Protein-2 (TIN2).[23,28,29] TIN2 protein is the central hub of the shelterin complex. TIN2 directly binds to and consequently stabilizes the TRF1 through two distinct mechanisms. First, TIN2 protects TRF1 from tankyrase 1-mediated poly(ADP-ribosyl)ation, which in turn ensures TRF1's association with telomeres.[30] Second, TIN2 competes with SCFFBX4 for binding to TRF1, thus preventing TRF1 from ubiquitin-dependent proteolysis.[22] Because of the important functions of TRF1 and TRF1-recruited TIN2 protein in telomere maintenance, the generation of small molecular compounds which bind to TRF1 and interfere with its coupling to TIN2 offers a potential tool to dissect the molecular mechanism of TRF-1-TIN2 interactions and may become a tool for destabilizing the whole shelterin complex, thus breaching cancer's survival strategy. This shelterin-based strategy has started to be explored also by other groups.[9,18,24]

Since both TRF1 and TRF2 proteins interact very similar with TIN2 as it was shown in experimental and in silico studies we focused only on modulation of TRF1-TIN2 interaction.[10,30,31] Crystal structure of TRF1 complexed with a TIN2 peptide (amino acids 256–276) has been solved and indicated that their interaction is mainly mediated by the TRFH domain of TRF1 and a 20-mer TRF1 binding motif in TIN2 (TIN2 TBM).[30] Using TIN2 TBM, (amino acids 256–276), as a targeted domain, and our previous molecular modeling of this molecular target we designed N-terminally tripeptides to inhibit TRF1-TIN2 interactions.[10] Here we report on the design, synthesis, and biochemical/biological evaluations of TIN2 TBM interfering model of modified peptides in a set of complementary approaches that collectively prove modulation of TRF1-TIN2 binding and promise a new approach against cancer. However, our just two new molecules can be regarded only as a models and used more as a proof of concept rather than established drug candidates.

Results and Discussion

Synthesis of PEP1 and PEP2

Two modified peptides molecules, PEP1 and PEP2, were synthesized following the general pathway shown in Figure 1. To confirm the structures of the obtained compounds for further studies elemental analysis (CHNS), mass spectrometry (MS), spectroscopic (^{1}H NMR) were performed. The MS and NMR plots were shown as Figures S2 and S4, respectively, together with elemental analysis information.

More detailed description of the synthesis of the modified tripeptides PEP1 and PEP2, and their purification can be found in the Supporting Information file (Figures S1–S3).

In silico studies

Although our major aim was TRF1 protein, we performed the in silico studies for both TRF1 and TRF2 since both of them interact with the TIN2 peptide and whether interactions of TIN2 share similarities towards TRF1 and TRF2. Although the crystal structures of the TRFH domains of the TRF1 and TRF2 proteins have almost identical molecular structures, they share only 27% sequence identity of amino acids.[10,31–33] The TRF proteins lack hetero-interactions with each other; both bind to the double-stranded telomeric DNA as homodimers. Despite similar docking sites for DNA, due to significant sequence differences, they slightly differentially associate with other proteins of the shelterin complex, but the docking site for TIN2 is rather similar for both cases.[31] It has been found that the required short motif of TIN2 for docking to the TRF1 is FxLxP while for docking to the TRF2 motif is YxLxP (Figure 2).[34–37] Considering the importance of such FxLxP and YxLxP motifs by TRF proteins, we used such motifs to design new peptide-like molecules using fragment-based approaches. For the designing of novel peptide-like molecules, we extracted the core motifs from TIN2 peptide showing approach a high occupancy and/or stable interactions with either of the TRF proteins by molecular dynamics (MD) approach (Figure 2).[10,32] In particular the FNL motif of TIN2 was important for interaction with one hot spot of TRF protein, a crucial one from structural point of interaction.[10]

Applying the fragment-based approach we added different functional groups at the N-terminal of the FNL motif (Figure 3; left panel) of the TIN2 peptide. These R1 fragments were selected within Molecular Operating Environment (MOE software: Chemical Computing Group Inc., Montreal, QC, Canada) approach based on the best scores concerning binding. In the first approach many potential R1 fragments were selected in automatic way by MOE. However, since the chemical structure

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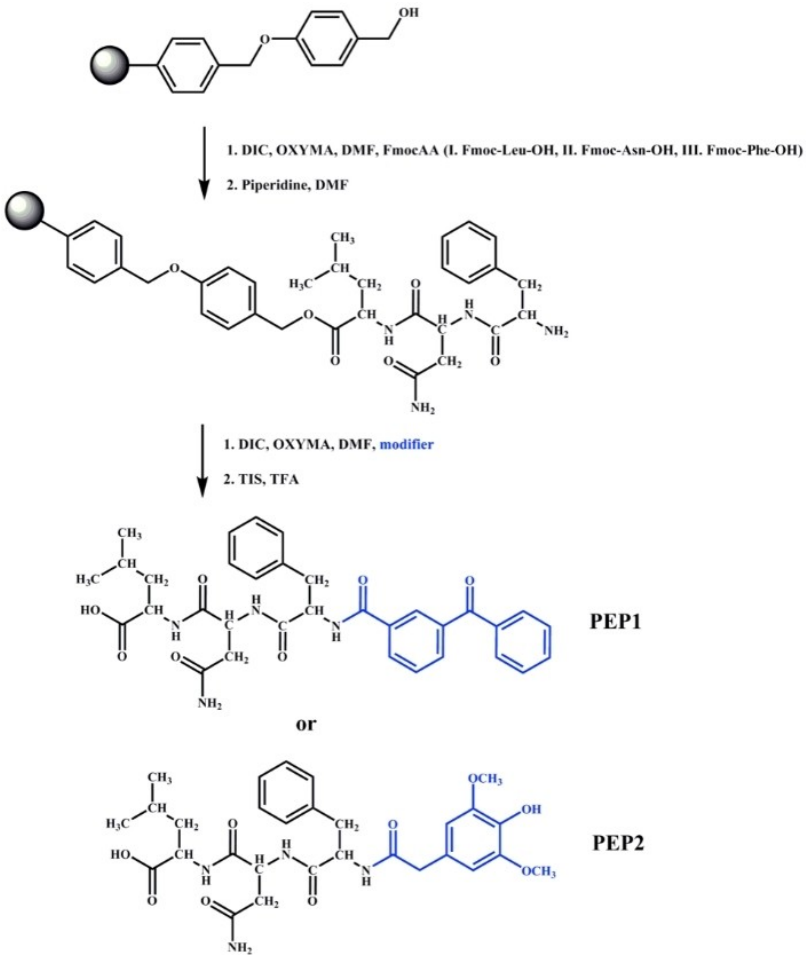

Figure 1. Solid-phase peptide synthesis using the Fmoc/tBu strategy. Synthesis was carried out on a (PHB)-Wang resin. After attaching the first amino acid to resin by standard ester coupling, standard Fmoc peptide synthesis was conducted using the N,N′-diisopropylcarbodiimide, and OxymaPure. Deprotection of all of the side-chain-protected peptide was conducted using a 20% piperidine solution in N,N′-dimethylformamide. After attachment of the last amino acid, modifiers were attached, for PEP1 3-benzoylbenzoic acid, for PEP2 3,5-dimetoxy-4-hydroxyphenyl-acetic acid. PEP1 and PEP2 were cleaved from the resin using the TFA method. Each elongation step was controlled by the chloranil test. This is a test for free amino groups. A positive test result leads to repeated acylation. In our case it was always negative. The HPLC, and MS confirmed correctness of the peptide synthesized (Figures S**1**-**S2**). In our case the modification of the peptide was straightforward with carboxylic acid using the same procedure.

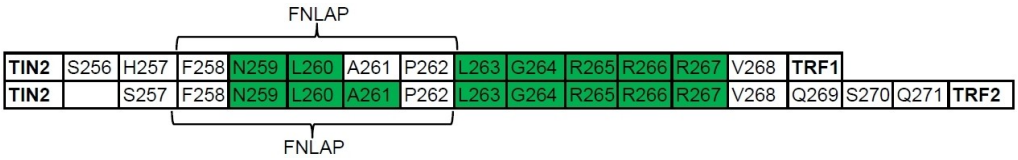

Figure 2. Residues making high occupancy (≥ 20% of time in MD simulation) interactions were traced from our previous study of monomer and dimer TRF1/2 systems.[10,32] FxLxP and YxLxP motifs are labeled, and the green color represents residues showing high occupancy (≥ 20%) interactions from TIN2 peptide with TRF proteins.

of these fragments were very unusual and synthesis of originally selected R1 substituents at the N-terminus (Figure 3; left panel) has not been possible from the chemical point of view we decided to use another more rational way. We designed an alternative set of N-terminally modified tripeptides as shown in Figure 3 (right panel) based on the same FNL tripeptide. New R1 substituents can be regarded as isosteric replacement. Therefore, an isosteric replacement of two modified peptides, based on chemical accessibility, has been proposed (Figure 3, right panel). Dataset of new compounds was screened against the respective TRF1/2$_{TRFH}$ proteins; predicting the peptide-protein binding affinity (Figures 3 and 4, respectively). Since both compounds have quite similar binding properties towards TRF1 and TRF2 we decided to perform experimental studies only with TRF1, especially since TIN2 binds to a similar site of both TRF proteins.

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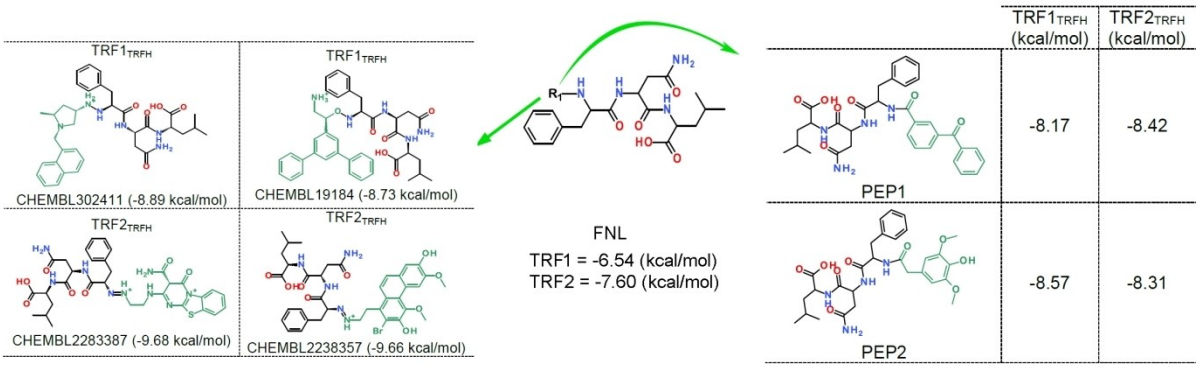

Figure 3. FNL extended at position R_1; fragments to FNL peptide added by applying the fragment-based approach to design novel peptides towards TRF1/2_{TRFH} protein, names of fragments from CHEMBL library are given (left panel). PEP1 and PEP2 modified tripeptides designed as isosteric analogues of original FNL–R1 substituents obtained by MOE (right panel).

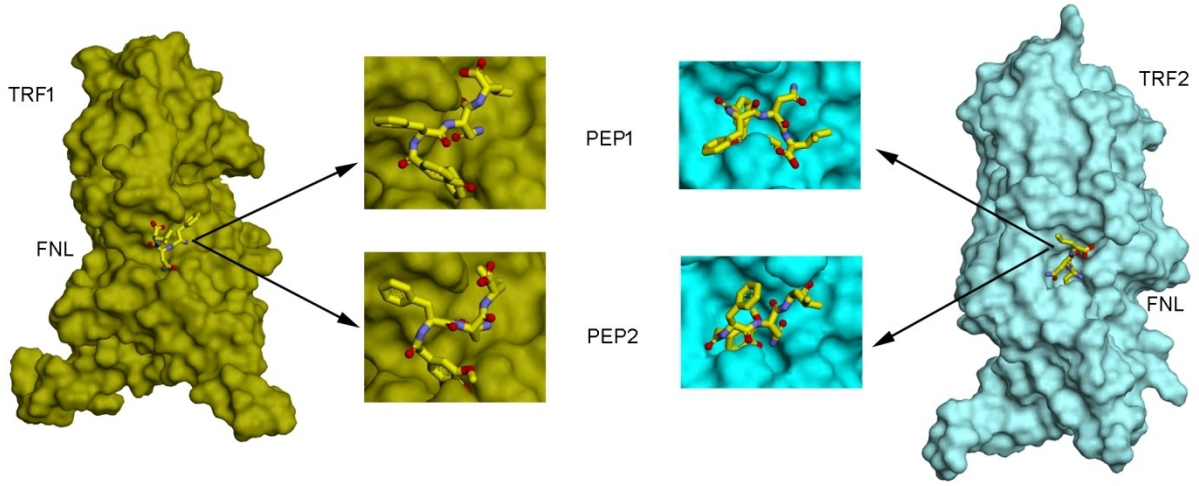

Figure 4. Docked conformation of peptides PEP1 and PEP2 against $TRF1_{TRFH}$ and $TRF2_{TRFH}$ protein.

SPR

To check the interaction pattern for PEP1 and PEP2 with TRF1 protein the SPR analysis (as more selective than for instance ITC) was performed and the results are shown in Figure 5. The increasing concentrations of each peptide were injected and flowed over the surface of the sensor chip with covalently immobilized TRF1 protein. A significant increase in the response was detected when PEP1 was injected (Figure 5A). In the case of PEP2, only a slight increase in the response was detected (Figure 5B). For interaction between PEP1 and TRF1 protein, the kinetic constants were calculated, using the 1:1 binding model (Figure S4 of Supporting Information). The association rate (k_a) was calculated as $1.18 \cdot 10^2\ M^{-1}s^{-1}$ ($\pm 1.49 \cdot 10^1\ M^{-1}s^{-1}$), dissociation rate ($k_d$) as $1.56 \cdot 10^{-3}\ s^{-1}$ ($\pm 1.01 \cdot 10^{-4}\ s^{-1}$) and equilibrium dissociation constant (K_D) value was $1.34 \cdot 10^{-5}$ M ($\pm 2.29 \cdot 10^{-6}$ M). Since the interaction between PEP2 and TRF1 protein was very weak (Figure 5B) the calculation of kinetic constants was not feasible.

As a control, instead of compounds the TIN2 peptide (RHFNLAPLGRRRVQSQWASTR) was used (Figure 5C). The association rate (k_a) was calculated as $1.90 \cdot 10^3\ M^{-1}s^{-1}$ ($\pm 5.49 \cdot 10^2\ M^{-1}s^{-1}$), dissociation rate ($k_d$) as $3.15 \cdot 10^{-2}\ s^{-1}$ ($\pm 2.73 \cdot 10^{-2}\ s^{-1}$), and equilibrium dissociation constant (K_D) value was $1.46 \cdot 10^{-5}$ M ($\pm 1.09 \cdot 10^{-5}$ M).

In Figure S5 of the Supporting Information the influence of PEP1 on TRF1 binding to TIN2 was shown. The biotinylated TIN2 peptide was immobilized on SA sensor chip. Next, the PEP1, TRF1 protein or a mixture of both flowed over the sensor chip surface and the obtained response was detected. The sensorgraphs showed the mean value from four experiments. However, no inhibition was observed what may suggest that either there is another place for binding PEP1 on the surface of TRF1 or our modified peptide is too short to occupy the whole TIN2 binding cleft and to completely prevent TRF1-TIN2 binding.

Cytotoxicity

Both PEP1 and PEP2 were not toxic to MCF7, MDA-MB-231, HCC116, A549, and HMEC cells with an IC50 (the concentration causing a 50% inhibition of viability compared to untreated cells) of ~100 uM (Figure 6).

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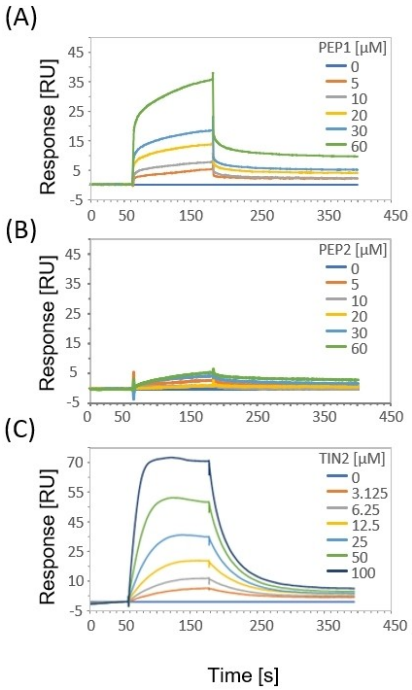

Figure 5. Analysis of PEP1, PEP2 and TIN2 interaction with TRF1 protein. The interaction of peptides, PEP1 (A), PEP2 (B) and TIN2 (C) with TRF1 protein was analyzed with surface plasmon resonance as described in Materials and Methods. The increasing amounts of PEP1, PEP2 (5, 10, 20, 30, 60 μM) and TIN2 (3.125, 6.25, 12.5, 25, 50, 100) were run over the surface of a sensor chip with immobilized TRF1 protein. The results are presented as sensorgrams obtained after subtraction of the background response signal from a reference flow cell and a control experiment with buffer injection. For each analyzed peptides, at least three kinetic experiments were performed.

Senescence and Lipid droplets

Replicative senescence (RS) has long been characterized as a proliferative arrest that occurs in normal cells after a limited number of population doublings, in a process largely depending on telomeres shortening and lowering of telomerase activity. In addition to RS, a number of factors can accelerate and/or trigger cell senescence, including various forms of stress - a phenomenon known as stress induced premature senescence or SIPS.[38] Exposure to different types of acute sub-lethal stresses such as oxidative stress and DNA damaging agents was shown to induce cellular senescence in different cell types at relatively short periods of time (ranging from 3 to 10 days) without or with modest telomere shortening.[39] The molecular biomarkers of senescent cells include senescence-associated β-galactosidase (SA-β-gal), p16INK4a, and p53.[40–42] To identify the possible senescence-involved cytotoxic activity, we performed a SAβ-gal assay. MCF7, A549 and HCT116 cells were plated in a 35 mm Peri dish on coverslips at low density for the logarithmic phase growth. The next day, the cells were treated with PEP1 and PEP2 at 100 μM concentration for five days. Etoposide (10 μM) was used as the positive control as a senescence inducing agent, and DMSO was used as the negative control. The large blue-stained senescent (SAβgal-assay-positive) cells were visible in the case of treatment of both peptides (Figure 7).

This observation confirmed, that compounds induced cell senescence, which can be related to the deregulation of the shelterin complex.[43,44] In human cells, the shelterin complex is composed of six proteins, including TRF1, TRF2, POT1, RAP1, TIN2, and TPP1.[45] The most important is that forming the whole 6 proteins complex requires the absence of steric hindrance between the components.[46] The TIN2 protein is involved in linking the double-stranded DNA binders TRF1 and TRF2 to the TPP1-POT1 heterodimer that deals with the telomeric single-stranded overhang.[47] Critical functions of TPP1 is the regulation of telomerase recruitment to telomeres via the interaction with telomerase reverse transcriptase (TERT), a catalytic part of the telomerase, and consequently for telomere maintenance.[48] Therefore, the used peptides might influence the recruitment of telomerase, which resulted in cellular aging visible in the experiment. It is interesting to note that PEP2, which is less active concerning interaction with TRF1 protein (as indicated in SPR experiments) is also less potent to promote senescence (Figure 7). This observation supports our proposed mechanism of action where both compounds appear not immediately cytotoxic but induce cellular stress leading to senescence rather than apoptosis. Our model of PEP1 and PEP2 activity assumes that disruption of TRF1-TIN2 interaction disrupts further shelterin intermolecular interactions leading to prolonged cellular stress.

Several studies reported an accumulation of lipid droplets in senescent cells compared to proliferating cells.[49–54] Lipid droplets are complex organelles with multiple functions that include modulation of nuclear processes, protein trafficking, membrane trafficking, and phospholipid recycling as well as metabolic regulation and storage of hydrophobic components.[55,56] Therefore, based on the previous result, the accumulation of lipid droplets in cells after PEP1 and PEP2 treatment was explored. Using the Nile red dye in MCF7 cells, we observed effects of PEP1 and PEP2 on lipid droplets (LDs) (Figure 8). The results show a clear increase in LD size in the cells after PEP1 or PEP2 treatment.

Telomere Dysfunction and Co-immunofluorescence

Telomere dysfunction-induced focus (TIF) assay allows efficient profiling of telomere dysfunctions in cells. As it is shown in Figure 9 treatment of cells MCF7 by PEP1 and PEP2 after 120 h lead to chromosomal aberrations. As we propose our compounds have ability to prevent protein TRF1-TIN2 interactions what may lead to non-functional behaviour of the shelterin complex followed by telomere unprotection and chromosomal aberrations. This cellular effect correlates with our in vitro results. Moreover, to study other effect in the telomeric region we applied co-immunofluorescence technique. Immunofluorescence is a technique to visualize the localization of specific molecule targets within cells using the specificity of antibodies. In this study, two compounds were used: PEP1 (100 μM) and PEP2 (100 μM), and the colocalization of γ-H2AX histone with TIN2 protein before and after MCF7 cells' treatment (after 120 h). The results were negative (Figure S1 of the Supporting

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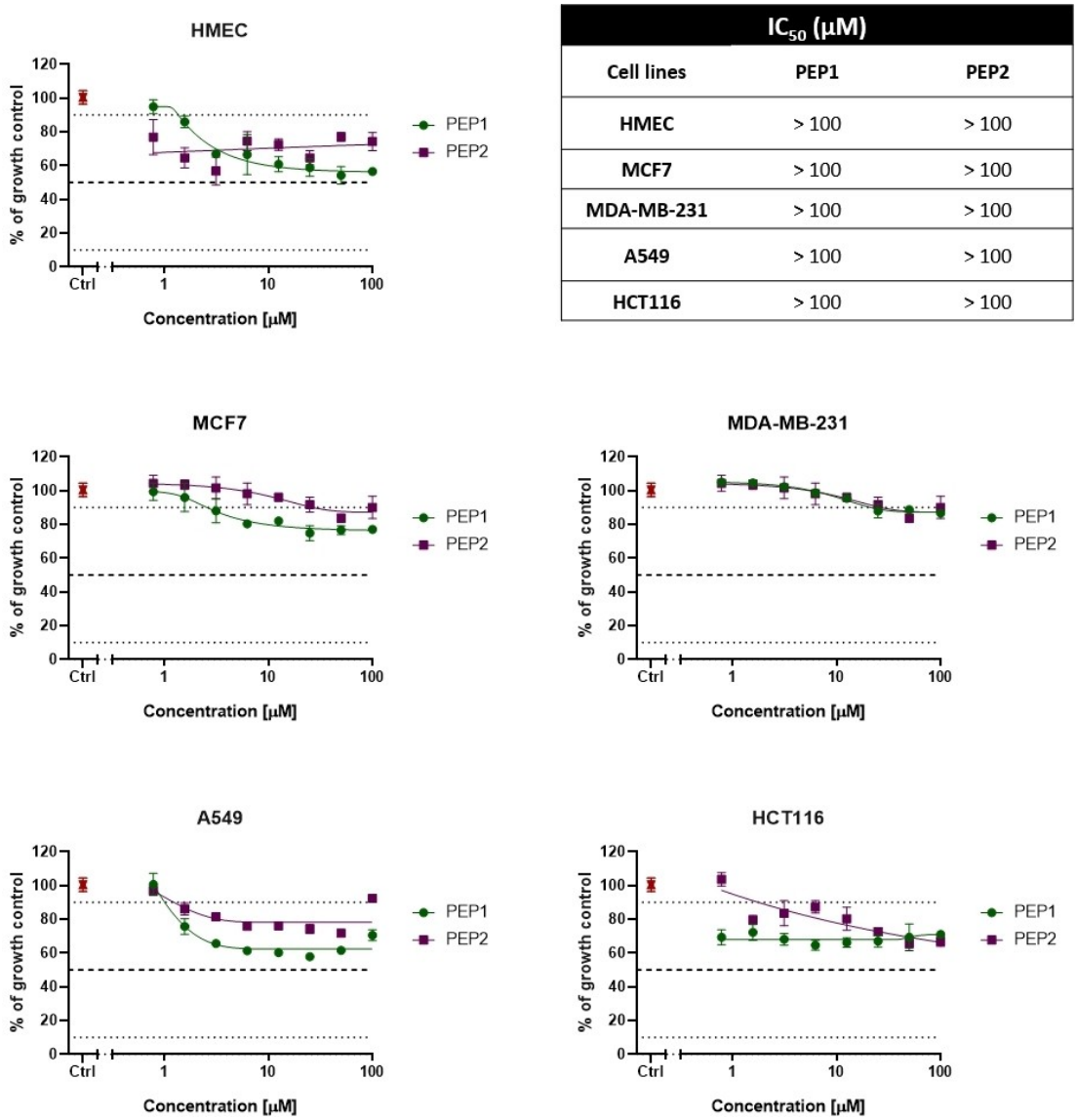

IC_{50} (μM)		
Cell lines	PEP1	PEP2
HMEC	> 100	> 100
MCF7	> 100	> 100
MDA-MB-231	> 100	> 100
A549	> 100	> 100
HCT116	> 100	> 100

Figure 6. Viability of HMEC, MCF7, MDA-MB-231, A549 and HCT116 cells measured by MTT assay after 72 h of incubation with PEP1 and PEP2.

Information) and TIN2 after cell treatments by our compounds remain in telomeric region as in a control experiment. However, this result does not contradict to our other observations and hypothesis of protein-protein inhibition by PEP1. Inhibition may prevent binding TIN2 to TRF1 but still other components of shelterin complex are present around what keeps TIN2 next to telomeres.

Conclusions

Combating cancer evolves into new strategies and involves novel targets whose search is extensively aided by in silico molecular modeling. New small molecules aimed to target telomer and its shelterin complex emerge as a novel promising strategy, ensuring high-precision interference. Telomeres normally get shortened after each cycle of cell duplication to ensure limitation of life span, however cancer cells often activate telomerase and hitchhike shelterin complex to enter quasi-immortalized state and become hyperproliferative. To breach this proliferation advantage of cancer cells over normal cells, herein, we interfered with telomere shelterin interaction between its two critically important components; TRF1 and TIN2. Our in silico modeled, designed and de novo synthesized small molecules of modified peptide, that mimic the core of TIN2 binding domain interfere with TIN2 binding to TRF1, as we observed in vitro. Modulating of TIN2/TRF1 coupling, demonstrated by us in the SPR analysis in a concentration series, especially in case of PEP1 modified peptide appeared not immediately toxic but on the other hand has triggered cell senescence. The results support our hypothesis about stepwise mechanism of action of PEP1 molecule, starting from interference with TIN2-TRF1 coupling and followed by prolonged cellular stress. One may expect such cellular response because

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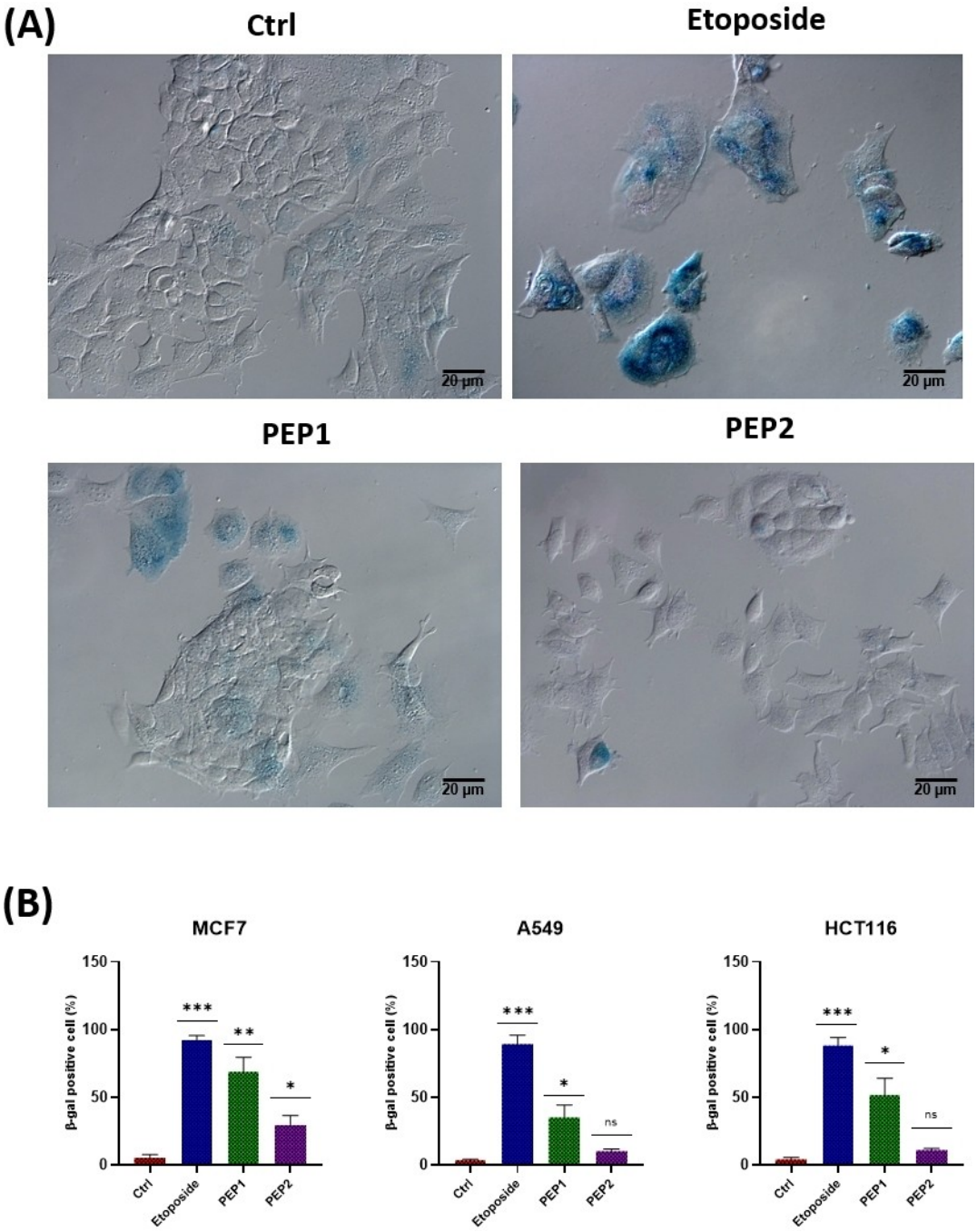

Figure 7. Senescence-associated β-galactosidase (SA-β-gal) staining. (A) Representative images of the MCF7 cell line after exposure to the following compounds: PEP1 (100 μM), PEP2 (100 μM) and etoposide (10 μM) after 120 h incubation. Ctrl denotes reference. Scale bars correspond to 20 μm. (B) Quantification of SA-β-gal positive cells for quiescent control, Etoposide, PEP1 and PEP2. The percentage of SA-β-Gal positive cells as an indication of SA-β-gal activity was quantified in n = 10 images per condition. Data presented as standard error mean bar and asterisks denote statistical significance (***–$P \leq 0.001$, **–$P \leq 0.01$, *–$P \leq 0.05$, ns–$P > 0.05$) by ANOVA test.

modulation of TRF proteins may not immediately cause harm for cells but instead induce cellular stress and result in senescence rather than apoptosis, as was demonstrated in our experiments. Moreover, from pharmacokinetic point of view, the accumulation of PEP1 and PEP2 in enlarged lipid droplets of treated cells promises prolonged depo-type release, usually an extra advantage among cancer chemotherapeutics and additionally limiting cytotoxicity of PEP1. Nevertheless, experiments performed at cellular level (TIF assay) support our in vitro observations that such modified peptides may lead to disfunction of telomeres and aberration of chromosomes. Modulation of TRF1/TIN2 binding by PEP1 small molecule proved by our study demonstrates the usefulness of in silico prediction and delivery of a new-lead of modified peptides class of anticancer compounds targeting one of shelterin protein. Since TRF proteins are relatively new anticancer targets further

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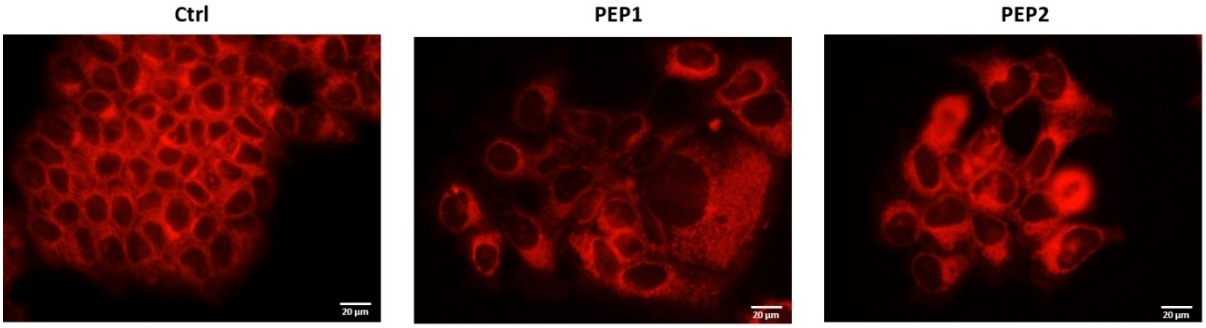

Figure 8. Detection of lipid droplets (Nile Red staining) in the population of cells of the MCF7 cell line after exposure to compounds PEP1 and PEP2 after 48 h of incubation. **Ctrl** denotes reference. Scale bars correspond to 20 µm.

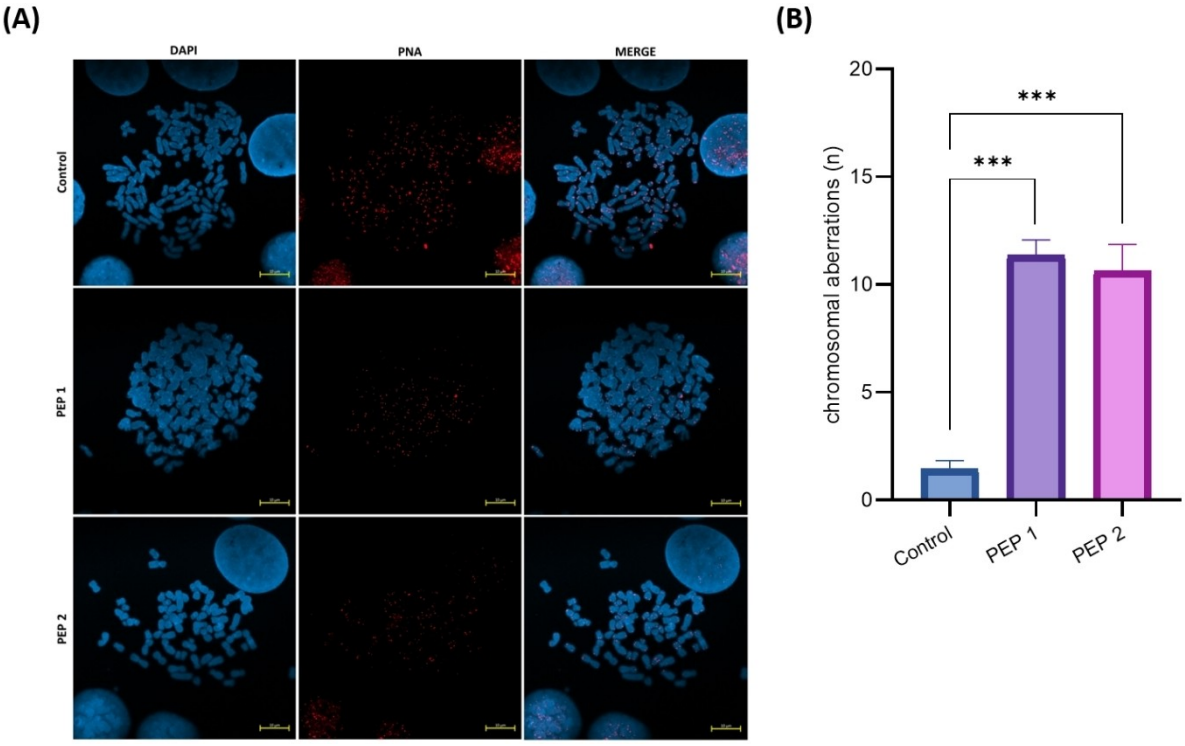

Figure 9. (A) Examples of metaphase spreads for MCF7 cells after exposure to the following compounds: PEP1 (100 µM), PEP2 (100 µM). Incubation time: 120 h. Telomers were identified by PNA telomere probe (red). DNA was stained by DAPI (blue). Scale bar equals 10 µm. (B) Quantification of telomere fusion. Data presented as standard error mean bar and asterisks denote statistical significance (***–$P \leq 0.001$, **–$P \leq 0.01$, *–$P \leq 0.05$, ns–$P > 0.05$) by ANOVA test.

studies are needed to answer the question how modulation or blocking these proteins influences preferentially the cancer cells in comparison with normal ones. Moreover using inhibitors of TRF, such as model modified peptide presented in our work, enables studying consequences of such cellular effects with high targeting precision.

Supporting Information

The *Supporting Information (SI)* file includes experimental information, the elemental analysis (CHNS), HPLC spectra, mass spectrometry (MS), and spectroscopic (^{1}H NMR), both methodology of the association (k_a), dissociation rate (k_d) and equilibrium dissociation constant (K_D) calculation, and a figure of influence of PEP1 on TRF1 binding to TIN2 from the SPR experiment. Additional references cited within the Supporting Information.[57–62]

Author Contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

Wioletta Brankiewicz – biological studies in part, cytotoxicity, senescence, analysis of telomere dysfunction and Co-immunofluorescence experiments – writing and reviewing

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manuscript in part. **Umesh Kalathiya** and **Monikaben Padariya** - in silico studies, – writing and reviewing manuscript in part. **Katarzyna Węgrzyn** - SPR studies – writing and reviewing manuscript in part. **Maciej Prusinowski, Joanna Zebrowska, Agnieszka Zylicz-Stachula**, and **Piotr Skowron** – designing and production of TRF1 – writing and reviewing manuscript in part. **Marek Drab** – supervised biological studies partially in particular lipid droplets experiments – writing and reviewing manuscript in part. **Mariusz Szajewski, and Maciej Ciesielski** – conceptualization in part of biological experiments, – writing and reviewing manuscript in part. **Małgorzata Gawrońska** – synthesis of PEP1 and PEP2 – writing and reviewing manuscript in part. **Anoop Kallingal** – telomere dysfunction and Co-immunofluorescence experiments. **Mariusz Makowski** – conceptualization of PEP1 and PEP2 synthesis and supervision of their synthesis – writing and reviewing manuscript in part. **Maciej Bagiński** – conceptualization, supervision of the whole manuscript – writing and reviewing manuscript in part.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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