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Research Article

Directed Blocking of TGF-β Receptor I Binding Site Using Tailored Peptide Segments to Inhibit its Signaling Pathway

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Background: Transforming growth factor (TGF)-β isoforms play crucial roles in diverse cellular processes. Therefore, targeting and inhibiting TGF-β signaling pathway provides a potential therapeutic opportunity. TGF-β isoforms bind and bring the receptors (TβRII and TβRI) together to form a signaling complex in an ordered manner.
Objectives: Herein, an antagonistic variant of TGF-β (AnTβ) has been designed and prepared to inhibit the formation of signaling complex and consequently its signaling pathway. This TGF-β homodimeric variant contains intact TβRII binding sites and blocked TβRI binding sites by substituting three peptide segments. So, AnTβ could only bind to TβRII, but prevent binding and recruitment of TβRI to form a signaling complex.
Materials and Methods: A reliable model of AnTβ was built and refined using molecular dynamics (MD) simulation, followed by investigating the interactions of AnTβ with the receptors using *in silico* docking studies. After expression of disulfide-linked AnTβ in a SHuffle strain and purification of the protein using affinity chromatography, its biological activity was evaluated using mink lung epithelial cells (Mvl Lu).
Results: No meaningful significant changes in AnTβ structure were observed when compared with the native protein. Based on the docking analysis, AnTβ binds to TβRII similar to TGF-β and its binding to TβRI was diminished considerably which was consistent with our design purpose. Cell-based bioassay indicated that AnTβ could modulate TGF-β-induced cell growth inhibition.
Conclusions: Our analysis suggests that the antagonistic potency of AnTβ can be used as an anti-TGFβ signaling factor in the future perspectives.
Keywords: Fibrosis; Protein engineering; Transforming growth factor beta (TGF-β); TGF-β antagonist

1. Background

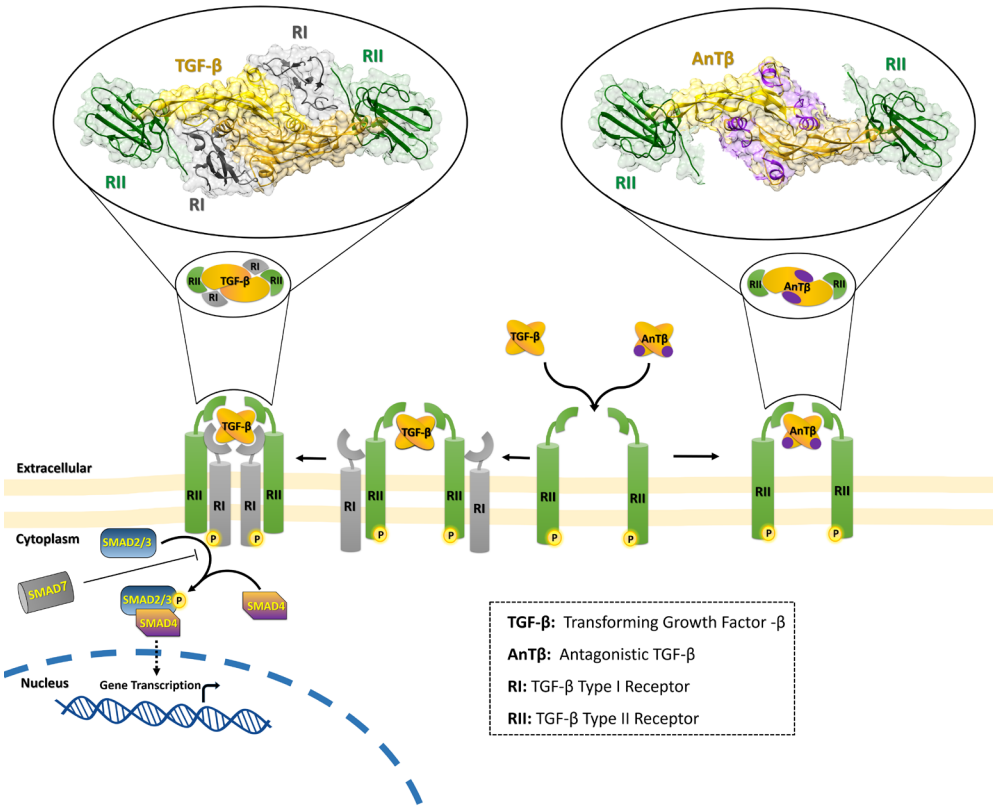
Transforming growth factor (TGF)-β isoforms are involved in the regulation of a wide variety of biological processes including cell proliferation, differentiation and expression of extracellular matrix proteins. However, dysregulation of their signaling has been implicated in various human diseases including cancer, fibrosis, autoimmune diseases and vascular disorders (1-3). The three TGF-β isoforms (TGF-β1, -β2, and -β3) in mammals, share high sequence identity (~ 71–79%) and structural similarity (root mean square deviations (RMSD) for backbone < 1.5 Å) and signal through the same receptors (4-7). Mature TGF-βs are ~25 kDa disulfide-linked homodimers, each monomer contains four intra-chain disulfide bonds (5, 6). TGF-β type I and II receptors (known as TβRI and TβRII, respectively) have the same overall domain structures, including an extracellular ligand-binding domain, a single transmembrane helix, and a cytosolic serine-threonine kinase domain. Signaling complex formation occurs when dimeric TGF-β1 or -β3 binds to two TβRII with high affinity. Then, TGF-β:TβRII complex recruits two TβRI, forming a heterotetrameric complex of receptors (1, 8, 9). TGF-β2 lacks two key arginine residues that are present in TGF-β1 and TGF-β3 which facilitate the high affinity interaction with TβRII; therefore, TGF-β2 requires a co-receptor (β-glycan or TβRIII) in order to form a signaling complex (8, 9). The ternary complex formation leads to the transphosphorylation of the TβRI (C-terminal kinase domain) by the adjacent TβRII (serine–threonine kinase). Type I kinase in turn phosphorylates nuclear translocating Smad proteins, leading to the activation of further downstream signaling events (10). Receptor-

regulated Smad proteins (R-Smads), Smad2 and Smad3 are TGF-β downstream signaling regulators that shuttle between the cytoplasm and nucleus. An inhibitory Smad (I-Smad), Smad7, can inhibit the phosphorylation of Smad2 or Smad3. Upon phosphorylation, p-Smad2/3 combines with common Smad (co-Smad), Smad4; which will later on translocate to the nucleus as a complex. Once in the nucleus, the Smad complex interacts with various transcription factors to activate or repress the expression of various genes in a cell-specific manner (1, 11, 12).

Disruption or dysregulation of the TGF-β signaling pathway promotes several human diseases. For instance, TGF-β hyperactivity leads to fibrotic disease of the kidney, liver and lung followed by tissue injury or disease progression due to the accumulation of extracellular matrix proteins (13). Furthermore, TGF-β was found to be involved in cancer development and progression. TGF-β signaling has been shown to play a dual contrasting behavior exerting either a tumor suppressor or a pro-oncogenic activity. In normal cells, TGF-β can act as a tumor suppressor and a potent inhibitor of cell proliferation by inhibiting G1 to S cell cycle progression and stimulating apoptosis. However, in cancer, TGF-β reverses its effect through promoting tumor invasion and metastasis by affecting both tumor cells and their surrounding microenvironment (13-16). As a promising strategy for the treatment of cancer, fibrotic disease, and several other diseases, inhibiting TGF-β signaling has become a pharmaceutical area of intense investigation. Accordingly, TGF-β isoforms and their receptors are potentially considered as major therapeutic targets (17-19). Several TGF-β signaling inhibitors, including antibody against the receptor and ligand (20), soluble receptors (21, 22), small molecules with kinase inhibitor activity (21, 23-26), antisense oligonucleotides (27, 28) and antagonistic monomer of TGF-β (29) have been developed so far. Although some of these inhibitors have shown promising activity in clinical trials, even phase III, none of them is currently approved by FDA (Food and Drug Administration) (30).

2. Objectives

Crystal structures of human TGF-βs:TβRI:TβRII ternary complex have provided a detailed information about the binding of TGF-βs to TβRI and TβRII (7, 31) and given a clear insight about the function of this signaling complex. Accordingly, in the present study, a novel antagonistic TGF-β variant was designed and produced based on the structural information. Since the antagonistic variant of TGF-β (AnTβ) contains intact receptor II and blocked receptor I binding sites, it can only bind to TβRII (**Scheme 1**), thereby inhibiting receptor heterotetramerization and blocking TGF-β signal transduction.



Scheme 1. Inhibition mechanism of receptor heterotetramerization and TGF-β signaling pathway by the antagonistic TGF-β (AnTβ).

3. Materials and Methods

3.1. Materials

Isopropyl thio-β-D-galactoside (IPTG) was obtained from Invitrogen (Carlsbad, CA, USA). Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography was provided by Qiagen (Qiagen, Hilden, Germany). *E. coli* BL21 (DE3) and SHuffle T7 *E. coli* K12 were obtained from Novagen (Madison, WI, USA) and New England Biolabs Inc. (Beverly, MA, USA) respectively. Cell culture medium was obtained from Bioidea company (Bioidea Company, Tehran, Iran) and fetal bovine serum was purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Mv1Lu cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and all other chemicals were obtained from Merck (Merck, Darmstadt, Germany).

3.2. Designing and Modeling of TGF-β Antagonist

The crystal structures of TGF-β3 (PDB ID: 1TGJ), ternary complexes of TGF-β3:TβRII:TβRI (PDB ID: 2PJY) and TGF-β1:TβRII:TβRI (PDB ID: 3KFD) were retrieved from the Protein Data Bank (PDB). The Contact Finder (CFinder) server (http://bioinf.modares.ac.ir/software/nccfinder/) has been used to recognize the residues that are involved in the interactions between TGF-β, TβRI and TβRII, also the residues which play role in TGF-β dimerization. This server uses protein complex pdb file as an input and finds the residues involved in protein-protein interactions according to the differences of accessible surface area (delta-ASA) between the complex and any of the chains that are selected.

TGF-β segments which are involved in binding to TβRI should be replaced by the appropriate peptide fragments, that have a similar geometry but different physicochemical properties. Candidate sequences were selected using the ProDA (Protein Design Assistant) server (http://bioinf.modares.ac.ir/software/proda/) (32). This web-server proposes a list of distinct fragments through searching in the database of more than 500 million protein segments using the input parameters. The criteria used to search suitable fragments as follows: number of amino acid residues, amino acid sequence pattern, distance between the two ends of the fragments, secondary structure, polarity and accessibility patterns of amino acid residues. The fragments among the candidate sequences were selected by considering the amino acid content. After that, selected fragments were replaced in TGF-β3 sequence. The 3D structure of the designed AnTβ was built based on this sequence and the structure of TGF-β3 and dimeric TGF-β1 using MODELLER (version 9.17) (33) and 100 models were generated. The best model was chosen with the lowest MODELLER objective function score and also the stereochemistry of the model was checked using PROCHECK program (34).

3.3. Molecular Dynamics Simulations

Molecular dynamics (MD) simulations of the native dimeric TGF-β3 and AnTβ were performed using GROMACS 5.0.7 for a period of 40 ns with a Gromos96 force field (35). Each structure was solvated in a solvation box using a simple point charge water model (SPC) (36), with at least 10 Å distance between the protein and the edges of box. Systems were neutralized by adding Cl^- and Na^+ ions that were randomly replaced with water molecules. The systems were initially relaxed and any bad contacts between atoms were removed through steepest descent algorithm in an energy minimization (EM) step. Then, the minimized systems were equilibrated for 100 ps using canonical (NVT) and the isothermal–isobaric (NPT) ensembles. All simulations were performed at 310 K and 1 bar. Finally, the equilibrated systems were simulated for a period of 40 ns with a 2 fs time step to understand the possible effects of mutations on the structure of AnTβ. RMSD and radius of gyration for systems were investigated in order to determine the stability of MD simulations and the compactness of the proteins during simulations.

3.4. Molecular Docking

The refined AnTβ and TGF-β3 were docked against their receptors using ClusPro server (https://cluspro.org) (37). Since, TGF-β bind to receptors in a sequential manner, at first bind to TβRII and then TβRI, the docking was done in two steps. For initial docking, the simulated AnTβ and TGF-β docked against TβRII. In the next step, docking of the AnTβ:TβRII and TGF-β:TβRII binary complexes against TβRI were done. Finally, the docking output files were superimposed with the crystal structure of the corresponding protein complex (the crystal structure of TGF-β:TβRI:TβRII ternary complex) and the RMSD of the structures were computed by using the Swiss-PdbViewer (38). All molecular graphics images were drawn by the UCSF Chimera package (version 1.11) (39).

3.5. Construction and Expression of TGF-β and Its Antagonist

The coding sequences of mature human TGF-β3 and its engineered antagonist variant were synthesized by ShineGene Molecular Biotech, Inc. (Shanghai, China) and cloned between *Nde*I and *Bam*HI restriction sites of pET21a and pET28a expression vectors, respectively. The TGF-β3 and AnTβ genes were expressed in *E. coli*

BL21 (DE3), then TGF-β3 refolded from inclusion bodies into native folded disulfide-linked homodimers as previously described (40) with slight modifications. Despite all the efforts to refold the engineered antagonist variant monomers into AnTβ homodimer, the successful results were not achieved. For this reason, another approach was proposed to express the dimeric AnTβ in *E. coli* SHuffle T7 strain. pET28a containing the AnTβ gene was transformed into SHuffle competent cells, then several conditions were optimized including temperature of growth and expression, time of induction and concentration of the inducer (IPTG). SHuffle *E. coli* cells transformed with AnTβ gene were initially grown in LB medium at 30 °C on the shaker until the cells reached mid log growth phase (0.5 OD_{600}). Protein expression was induced with 0.1 mM IPTG and the growth temperature shifted to 15 °C for 24 h followed by harvesting the cells through centrifugation, lysis by sonication and collecting AnTβ in the supernatant. The AnTβ was purified using metal affinity chromatography on Ni-NTA agarose. For purification, washing buffer containing 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole and 5% glycerol and elution buffer containing 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 250 mM imidazole and 10% glycerol were prepared and used. All steps were done at 4 °C. The quality and purity of the protein samples were analyzed by reducing and non-reducing SDS-PAGE.

3.6. Growth Inhibition Assay

The biological activity of purified TGF-β3 and AnTβ were studied in the mink lung epithelial cells (Mv1Lu). This cell line is extremely sensitive to TGF-β that promotes a decrease in its proliferation level. The cells are cultured in a minimum essential medium with 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37 °C. For growth inhibition assay (41), Mv1Lu cells were trypsinized, washed with phosphate buffered saline (PBS), counted and seeded in 96-well plates. After cells attachment (3-4 h), the medium was replaced with fresh medium containing TGF-β, AnTβ or TGF-β/AnTβ at different concentrations. The cells were incubated for 24 h at 37 °C. Afterward, cell growth inhibition was analyzed using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. 5 $mg.mL^{-1}$ MTT solution was added to the media and the plates were incubated for 3 h at 37 °C. After that, media were replaced with 100 µL of DMSO and the absorbance of wells was determined at 570 nm.

4. Results

4.1. Designing Antagonistic TGF-β with Tailored Segments

Based on the accessible surface area (ASA) analysis, TGF-β residues that are involved in binding to TβRI and TβRII were identified. The segments which show the highest delta-ASA are considered as the most important residues in the binding process to type I and II receptors (**Fig. 1A**). Four regions of TGF-β interact with TβRI where two of them commonly with TβRII. Therefore, in order to design the homodimeric TGF-β antagonist (AnTβ), two important regions comprising ^{1}ALDTNY^{6}, ^{49}PYLRS^{53} and ^{57}THSTVLGL^{64} segments were finally selected (**Fig. 1B**) to be replace by the appropriate candidate sequences.

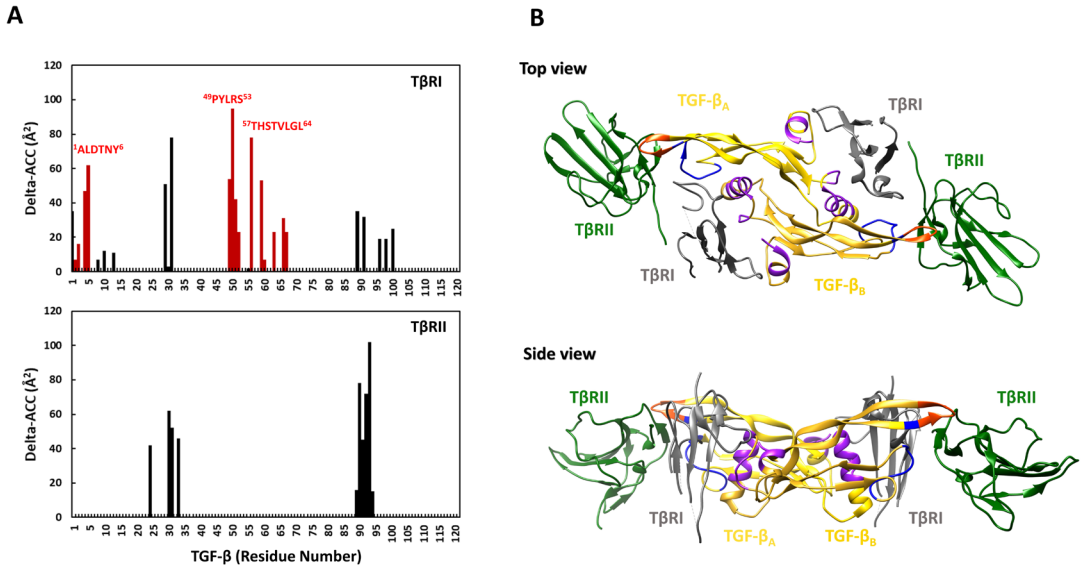
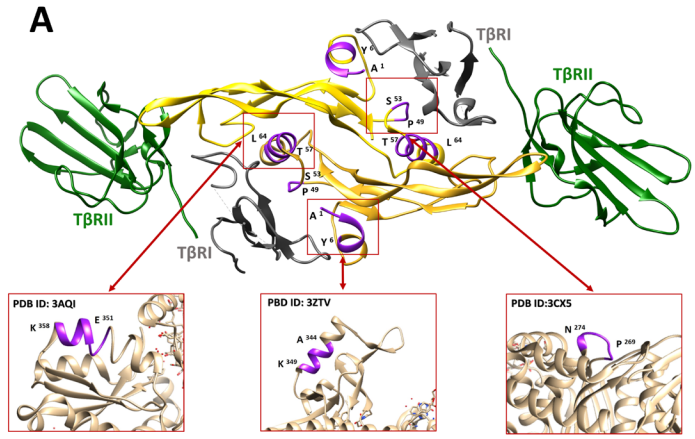


Fig 1. TGF-β/receptor interactions in TGF-β1 ternary complex. **(A)** Analysis of TGF-β binding sites to TβRII and TβRI. **(B)** Crystal structure of TGF-β1 (yellow) in the ternary complex with its receptors (PDB ID: 3KFD), TβRI (gray) and TβRII (green) showed in top and side views. Segments of TGF-β which are recognized by TβRI, TβRII and TβRI:TβRII complex are shown with violet, orange and blue colors, respectively.

Candidate sequences were chosen after considering structural criteria through searching in the ProDA server. These segments should be geometrically similar in order to avoid considerable changes in the protein structure and the binding affinity to TβRII, but should have different amino acid composition to inhibit AnTβ binding to TβRI (**Table 1**). The segment ^{1}ALDTNY^{6} which is an exposed α-helix, was replaced by ^{344}ALDTLK^{349} from 3ZTV pdb structure. The exposed turn segment ,^{49}PYLRS^{53}, was substituted by ^{269}PVNSPN^{274} from 3CX5 pdb structure. Finally, ^{57}THSTVLGL^{64} α-helix was replaced by ^{351}EYSQVLAK^{358} from 3AQI pdb structure (**Fig. 2A**). Eventually, these fragments were embedded in TGF-β3 sequence. Structure-guided sequence alignment of AnTβ and TGF-β isoforms have been illustrated in **Figure 2B**.

Table 1. Criteria for selection of the substituted segments in TGF-β.

TGF-β segments	Criteria for Selection				Substituted Segments
	Number of Amino Acids	Distance Between Two Ends (Å)	Secondary Structure	Surface location	
^{1}ALDTNY^{6}	6	6.5 - 9	Helix	Expose	^{344}ALDTLK^{349} (from 3ZTV)
^{49}PYLRS^{53}	6	8.5 - 9.5	Coil or Turn	Expose	^{269}PVNSPN^{274} (from 3CX5)
^{57}THSTVLGL^{64}	8	10 - 11	Helix	Expose	^{351}EYSQVLAK^{358} (from 3AQI)



B

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TGF-β1   ALDTNYCFSS TEKNCCVRQL YIDFRKDLGW KWIHEPKGYH
TGF-β2   ALDAAYCFRN VQDNCCLRPL YIDFKRDLGW KWIHEPKGYN
TGF-β3   ALDTNYCFRN LEENCCVRPL YIDFRQDLGW KWVHEPKGYY
AnTβ     ALDTLKCFRN LEENCCVRPL YIDFRQDLGA KWVHEPKGYY
         ***:  ** .  :.***:* * ****::***  **:******

TGF-β1   ANFCLGPCPY IWS-LDTQYS KVLALYNQHN PGASAAPCCV
TGF-β2   ANFCAGACPY LWS-SDTQHS RVLSLYNTIN PEASASPCCV
TGF-β3   ANFCSGPCPY LRS-ADTTHS TVLGLYNTLN PEASASPCCV
AnTβ     ANFCSGPCPV NSPNADTEYS QVLAKYNTLN PEASASPCCV
         **** *.**       ** :*  **. **  * * ***:****

TGF-β1   PQALEPLPIV YYVGRKPKVE QLSNMIVRSC KCS
TGF-β2   SQDLEPLTIL YYIGKTPKIE QLSNMIVKSC KCS
TGF-β3   PQDLEPLTIL YYVGRTPKVE QLSNMVVKSC KCS
AnTβ     PQDLEPLTIL YYVGRTPIVE QLSNMVVKSC KCS
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Fig 2. Substituted segments in AnTβ. **(A)** TβRI binding site and the swapped segments. The segments of dimeric TGF-β that are only in contact with TβRI were recognized and replaced by candidate segments. **(B)** Structure-guided sequence alignment of TGF-β isoforms and their antagonist. The secondary structure elements are illustrated as cylinders and arrows for α-helices and β-strands, respectively. The mutated residues of AnTβ are shown in red and bold.

4.2. Building Model and Structural Refinement

The 3D structure of designed AnTβ has been modelled. The structure with the lowest MODELLER objective function was selected for molecular dynamics simulation. MD simulations were used to refine the AnTβ and TGF-β structures in a similar condition and compare them before the docking procedure. After simulations, the RMSD and radius of gyration values for the backbone atoms of TGF-β (**Fig.3A**) and AnTβ (**Fig. 3B**) were monitored relative to the starting structure during the MD production phase. RMSD curves showed that the backbone of TGF-β and AnTβ structures were stable and reaching equilibrium after 15 and 5 ns of the simulation respectively. For both systems, from the mentioned times until the end of the simulations, RMSD values have no significant deviation. Radius of gyration for the proteins during simulations also showed unmeaningful changes in the compactness of the proteins.

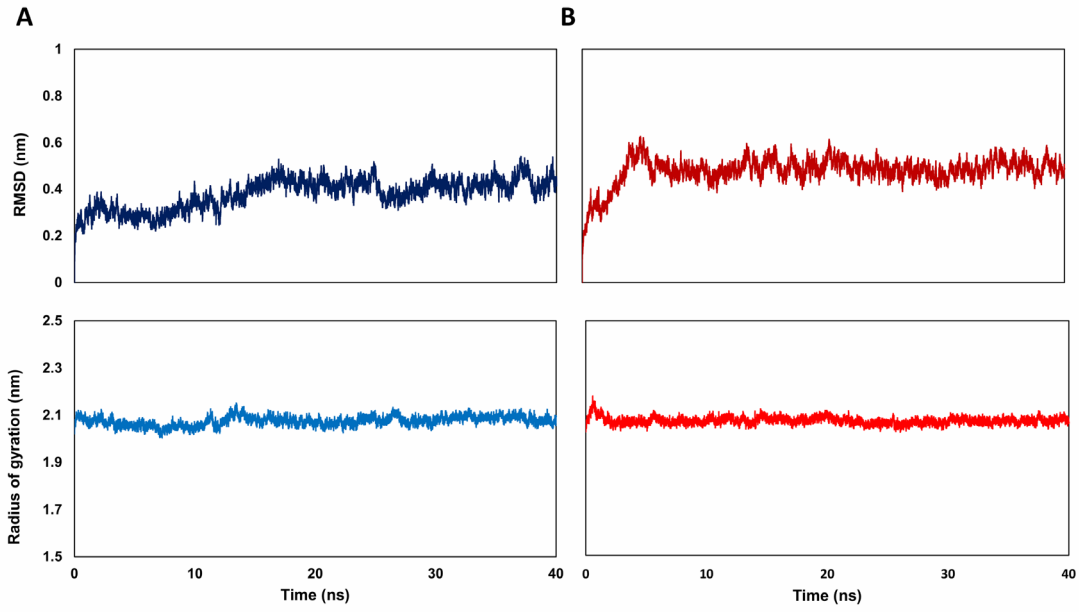

Fig 3. RMSD and radius of gyration of the proteins during simulations. RMSD (nm) and radius of gyration (nm) values of the backbone atoms of TGF-β **(A)** and AnTβ **(B)** structures with respect to the reference coordinate during 40 ns simulations.

4.3. Prediction of Binding Ability

The binding capability of the TGF-β and AnTβ to the type II and I receptors were analyzed. The docking results are presented in **Figure 4** and **Table 2**. The results showed that TβRII has the native pose in both of TGF-β/RII and AnTβ/RII binary complexes with the similar binding scores. In the next step, these binary complexes docked to TβRI. As illustrated in **Figure 4A**, in TGF-β ternary complex, docked TβRI (red ribbon diagram) was positioned similar to the receptor I in crystal structure (gray ribbon diagram). In AnTβ:RII/RI complex, the differences between TβRI states in crystal structure and docking result were clearly observed in **Figure 4B**. Obviously, AnTβ/RII binary complex lose the binding ability to TβRI.

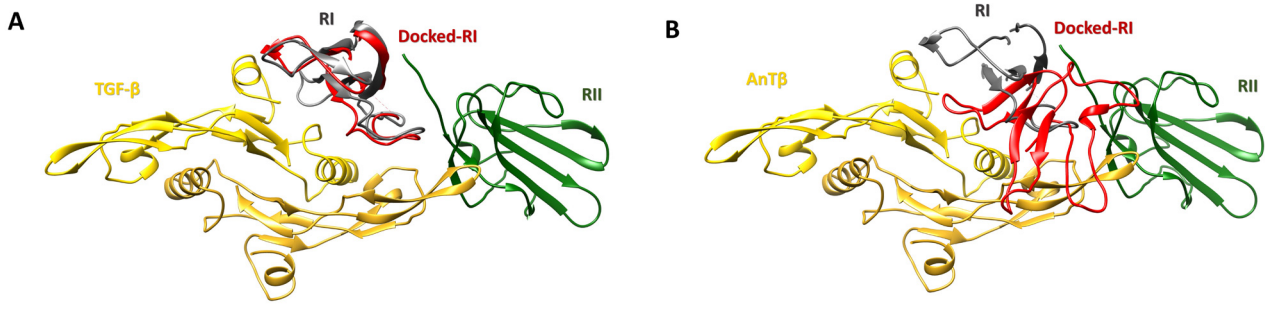

Fig 4. Comparison of TβRI binding state in TGF-β:RII/RI and AnTβ:RII/RI ternary complexes. The native ternary complex (PDB ID: 3KFD) superimposed to the docked structure, **(A)** TGF-β and **(B)** AnTβ. The corresponding TβRI position is shown as a gray ribbon diagram. TGF-β and AnTβ colored yellow and docked TβRI and TβRII colored red and green, respectively.

Table 2. The results from protein–protein docking obtained by ClusPro server.

Complex	Docking Component	Weighted Score	RMSD (Å) (vs. 3KFD)
TGF-β/RII	TGF-β and TβRII	-784.4	0.4
AnTβ/RII	AnTβ and TβRII	-671.9	0.6
TGF-β:RII/RI	TGF-β:RII complex and TβRI	-1135.7	0.2
AnTβ:RII/RI	AnTβ:RII complex and TβRI	-631.7	31.2

4.4. Preparation of TGF-β Antagonistic Variant

TGF-β and AnTβ were expressed in *E. coli* strain BL21 (DE3) and accumulated in the form of insoluble inclusion bodies. An obvious band with an expected size (~ 13 kDa) was observed using a reducing SDS-PAGE for both constructs (data not shown). The inclusion bodies were isolated, washed and denatured, then the monomers were renatured by dilution in folding buffer as previously described (40). TGF-β is a disulfide-linked homodimer, therefore, dimerization of the resulted constructs confirmed the refolding accuracy. The refolded constructs were determined using reducing and non-reducing SDS-PAGE. TGF-β was successfully refolded and revealed as a 25-kDa band under non-reducing conditions, however, the AnTβ remained as a monomer probably as a result of replacements and/or histidine tags (data not shown). As an another approach, SHuffle strain was used to produce AnTβ which is an *E. coli* protein expressing system capable of correctly folding proteins with disulfide bonds (42). SHuffle *E. coli* cells transformed with AnTβ gene were initially grown in LB medium at 30 °C on the shaker until the cells reached mid log growth phase (0.5 OD_{600}). Protein expression was induced with 0.1 mM IPTG and the growth temperature shifted to 15 °C for 24 h followed by harvesting the cells through centrifugation, lysis by sonication and collecting AnTβ in the supernatant. The soluble proteins in the supernatant were purified by Ni-NTA agarose affinity column chromatography. Purified protein loaded on SDS-PAGE under reducing and non-reducing conditions was monitored in the monomer and dimer form of AnTβ, respectively (**Fig.5**).

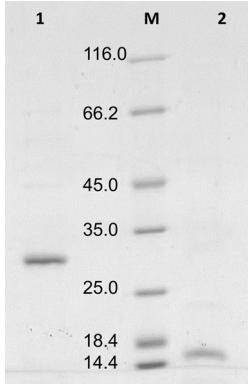

Fig 5. SDS-PAGE analysis of the purified AnTβ under reducing (lane 1) and non-reducing (lane 2) conditions. Lane M corresponds to the protein molecular mass marker.

4.5. Biological Activity of the AnTβ

Mv1Lu cells were used to evaluate the inhibitory effects of AnTβ on TGF-β-induced cellular responses. For growth inhibition assay, first, the cells were treated with the three concentrations of TGF-β (0, 5 and 10 pM) in the presence of three concentrations of AnTβ (0, 10 and 50 pM). Results showed that (**Fig. 6A**) in the absent of TGF-β ([TGF-β] = 0 pM), AnTβ had not effect on cell growth. In the presence of the TGF-β fixed concentrations (5 and 10 pM), increase in concentration of AnTβ led to decrease in the TGF-β inhibitory effect. In follow, growth inhibition assay was examined in the presence of various concentrations of AnTβ (0-50 pM). The dose-response curves, presented in **Figure 6B**, showed that AnTβ exhibited an efficient potency for reducing the growth inhibitory effect of TGF-β especially at lower concentrations of TGF-β, however, this result was not observed in samples untreated with TGF-β. Furthermore, more than 2-fold decrease in the inhibitory effect of TGF-β could be observed in the presence of AnTβ.

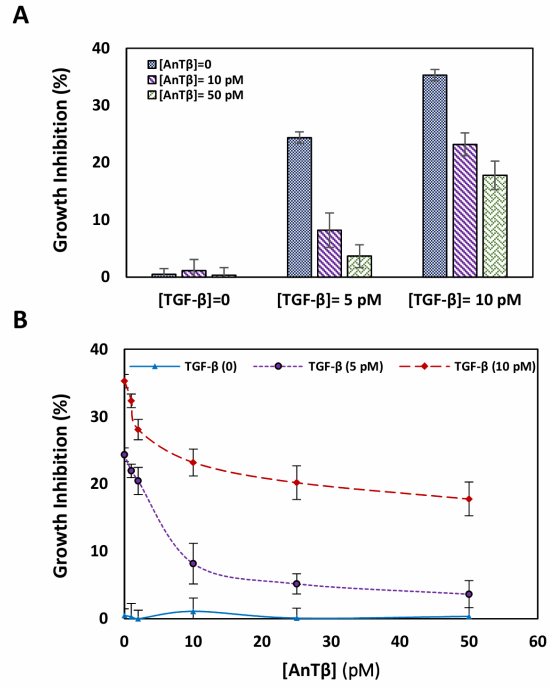

Fig 6. Inhibitory effects of AnTβ on growth of Mv1Lu cells (A and B). Growth inhibition of cultured cells was examined in the medium containing the fixed concentrations of TGF-β3 (0, 5 and 10 pM) in the presence of various concentrations of AnTβ (0-50 pM).

5. Discussion

TGF-β plays a crucial role in the regulation of several pathological processes including cancers as well as other non-cancerous diseases. TGF-β signaling inhibitors not only can be beneficial for treatment of cancers and fibrotic diseases, but also may be effective in treatment of autoimmune disorders such as multiple sclerosis, regulation of diabetes and obesity (17-19, 30). There are anti-TGF-β therapeutic strategies that have been previously developed including antisense oligonucleotides (ASOs) for downregulation of TGF-β expression (27, 28), neutralizing monoclonal antibodies (mAbs) for targeting ligands, receptors or associated proteins (20-22), ligand-competitive peptides and small molecule receptor kinases inhibitors (SMIs) (17, 21, 23-25, 30). Despite the beneficial effect of these inhibitors for treating a variety of human diseases, they are still amenable to improvement in terms of specificity, bioavailability, tissue penetration, size and so on. While kinase inhibitors show efficient inhibitory effect against a targeted kinase, their inhibitory function against other members of the kinase family remains a major challenge. In comparison with the drugs that function extracellularly, kinase inhibitors and other drugs that act in the cytoplasm have to pass through the plasma membrane in order to reach their targets leading to lesser bioavailability (23, 24, 26). Even though mAbs have high specificity and can overcome the drug resistance issue, their efficient tissue penetration is considerably limited by their large size (21, 22). The long-term tissue maintenance of TGF-β neutralizing antibodies causes side effects in the case of treating some types of carcinomas including squamous cell carcinoma and keratoacanthoma during clinical trials (17, 20, 22, 30). Accordingly, there is also an urgent need to design new TGF-β signaling inhibitors that can overcome these obstacles. Herein, a protein engineering strategy has been performed in order to design a homodimeric TGF-β antagonist. By applying this strategy, not only TGF-β signaling pathway would be specifically and efficiently abrogated, but also the drawbacks of the current inhibitors would be solved. This engineered protein specifically binds to the extracellular domain of TGF-β receptors and only inhibits TGF-β1, -β2, and -β3 signaling, but not other members of the kinase family. More accessibility of the antagonist to its target may result in its higher bioavailability relative to intracellular signaling pathway inhibitors. Due to its small size (one-sixth the size of conventional antibody), TGF-β antagonist would be expected to show a higher potency for tumor penetration and shorter tissue residence time than neutralizing mAbs. Given these valuable properties, TGF-β antagonist may be considered as a rational alternative to the other TGF-β signaling inhibitors.

TGF-β isoforms bind to TβRI and TβRII and assemble into a ternary complex in an ordered manner. Dimeric TGF-β forms a stable binary complex with two copies of TβRII and then this complex recruits two copies of TβRI (9, 43). The formation of a ternary complex (dimeric TGF-β:2TβRII:2TβRI) triggers a phosphorylation cascade whereby the TβRII phosphorylates the TβRI and consequently TβRI phosphorylates Smads (Scheme 1) (1). Tao Huang *et al*. (44) suggested that in the signaling complex, two TβRI:TβRII heterodimers initiated a signaling pathway individually in an autonomous manner. Based on this model, here, a TGF-β antagonistic variant in the dimer form was designed with an intact TβRII binding site and a mutated TβRI binding site in each chain. Therefore, the antagonist will bind to TβRII and prevents the recruitment of TβRI leading to the inhibition of heterotetrameric complex formation of the receptors and subsequently abrogates signal transduction.

Since all TGF-β isoforms have high structural similarity and signal through the same receptors (4-7), designing an antagonist based on the structure of each TGF-β isoforms is expected to be able to inhibit this signaling pathway. Superposition of TGF-β1 and TGF-β3 ternary complexes with unbound TβRII; and superposition of these ternary complexes and the unbound TGF-β1, TGF-β2 and TGF-β3, showed that there are no significant changes in TGF-βs and TβRII conformation upon complex formation (7). The binding potency of TGF-β to TβRII is isoform-dependent as TGF-β1 and -β3 have the highest binding affinities to TβRII and TGF-β2 has 1000-fold lower binding affinity (7-9, 45). In addition, TGF-β2 displays no receptor preference and may initially bind to either TβRI or TβRII and then recruit another receptor (7). Due to the low affinity of TGF-β2 for binding TβRII and a random-sequential assembly to its receptors, this variant was not considered as a template for designing AnTβ. Previous studies have shown that TGF-β2 and -β3 which have obtained through the expression of the mature monomers in *E. coli* as inclusion bodies were refolded into disulfide-linked dimers; which was not possible for TGF-β1 (40). For all these reasons, we designed a homodimeric TGF-β antagonist based on TGF-β3 amino acid sequence.

In an effort for determining the TGF-β receptor binding sites, the structural information of TGF-β/receptors complexes and accessible surface area (ASA) analysis were used, and the results were combined with previous

structural data (7, 9, 31). According to the difference of accessible surface area (delta-ASA) between unbound TGF-β and TGF-β/receptors ternary complex, TGF-β residues responsible for the interaction with the receptors were determined (**Fig.1**). The results have shown that three important segments of TGF-β, two α-helices ^{1}ALDTNY^{6} and ^{57}THSTVLGL^{64}, and ^{49}PYLRS^{53} with loop structure have a crucial role in TβRI binding, but not in TβRII binding and TGF-β dimerization. These TGF-β segments were substituted with the suitable segments to block the interaction with TβRI (**Table 1**). Finally, ^{1}ALDTNY^{6} and ^{57}THSTVLGL^{64} segments were substituted by ^{344}ALDTLK^{349} (from 3ZTV PDB structure) and ^{351}EYSQVLAK^{358} (from PDB ID: 3AQI), respectively, that are α-helices exposed with the same length and hydrophobicity pattern, but having different charge pattern. ^{49}PYLRS^{53} was replaced by ^{269}PVNSPN^{274} (from PDB ID: 3CX5) which is an exposed loop with the same distance between two ends and difference in the length and charge pattern. The longer length of the substituted loop may result in steric hindrance (**Fig. 2A**). The sequence of engineered TGF-β antagonist was determined (**Fig. 2B**) and its 3D structure was modeled. Molecular dynamics simulations of TGF-β3 and Antβ were performed in order to allow conformational relaxation of the structures in similar condition before the docking procedure. The results of the MD simulations studies showed no meaningful changes in AnTβ structure (**Fig. 3**). Additionally, results from the docking of the simulated TGF-β and AnTβ with type I and II receptors indicated that the binding score of TGF-β/RII and AnTβ/RII was similar (**Table 2**), and TβRII in both of these complexes was in the native position (**Table 2** and **Fig. 4**). Thus, it can be inferred that the antagonist was able to bind TβRII similar to TGF-β. Furthermore, TβRI in AnTβ:RII/RI complex bound at a different position in comparison with TGF-β:RII:RI. The results showed that AnTβ could bind to TβRII; however, the binary complex was not able to bind TβRI and forming a signaling complex.

The coding sequences of both TGF-β3 and AnTβ were synthesized and inserted in pET21a and pET28a expression vectors, respectively. TGF-β3 expression was obtained by *E. coli* BL21 (DE3) and refolded as described for TGF-β3 (40), however, the refolding wasn't successful for AnTβ. We then used SHuffel expression system to produce this protein in a disulfide-linked dimeric soluble form. The advantage of producing AnTβ in SHuffle cells is that the proteins remain soluble and can be extracted from the bacteria in their native form. As the N-terminus of the mature TGF-β is structurally flexible and accessible, the C-terminus is ordered and buried in the structure, thus the N-terminal part provides an appropriate site for tagging. In the case of N-terminal His-tag, the tag lies near the TβRI binding site and may block receptor binding and signaling (40) which is consistent with our aim. So, AnTβ containing His-tag at the N-terminus was expressed as a dimeric soluble form in the SHuffle strain and then purified using Ni-NTA affinity column chromatography and monitored by SDS-PAGE (**Fig.5**). TGF-β regulates proliferation in a cell-specific manner. In many cell types, including epithelial, endothelial, hematopoietic and immune cells, TGF-β strongly inhibits the growth of these cells (13, 46). In cancer cells, mutations in the TGF-β pathway confer tolerance to growth inhibition by TGF-β promoting uncontrolled cell proliferation (13, 47, 48). As widely reported, TGF-β isoforms are known to have anti-proliferative effects on mink lung epithelial (Mv1Lu) cells and TGF-β signaling inhibitors can neutralize these effects (49-51). According to our results, the growth inhibition effect of TGF-β on Mv1Lu cells was reduced in the presence of AnTβ especially at lower concentrations of TGF-β (**Fig.6A** and **B**). In the other word, while TGF-β acts as a cell growth inhibitor in these cells, AnTβ reduces its effect. Finally, the results indicated that AnTβ is efficient for modulating TGF-β-induced cell growth inhibition. Therefore, it is possible that the antagonistic potency of AnTβ may allow its future use as an anti-TGF-β factor, however many questions have been yet unanswered about their interactions or conformations and different *in vitro* and *in vivo* assays should be employed in order to confirm our suggestion.

6. Conclusion

In summary, TGF-β signaling inhibitors may be efficacious in some clinical applications, mainly in autoimmune disorders and in desperate cases such as end-stages of cancer or some diseases that are more lethal than many cancers such as idiopathic pulmonary fibrosis (IPF). Designing a homodimeric TGF-β antagonist with intact TβRII and mutated TβRI binding sites, which binds to TβRII and does not recruit TβRI, leading to inhibition of TGF-β signaling. This antagonist competes with TGF-β isoforms for binding TβRII and antagonizes its action by inhibiting the interaction between TGF-β and its receptors on the extracellular surface. Both *in silico* analysis (structural information, MD simulations and docking) and *in vitro* experiment (proliferation assay) confirmed the efficacy of AnTβ. Hence, the antagonistic potency of AnTβ may allow its future use as an anti-TGF-β factor and may exhibit further therapeutic opportunities.

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Author contributions

Sepideh Sepehri performed the bioinformatics studies and the experiments. Reza H. Sajedi and Seyed Shahriar Arab planned the experiments, conceived the study and analyzed the data. Seyed Shahriar Arab contributed in the bioinformatics studies. Mehrdad Behmanesh helped in the molecular biology techniques. Sepideh Sepehri, Reza H. Sajedi and Seyed Shahriar Arab discussed the results, contributed in writing and preparation of the manuscript.

Conflict of Interest

There is no conflict of interest with this study.

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