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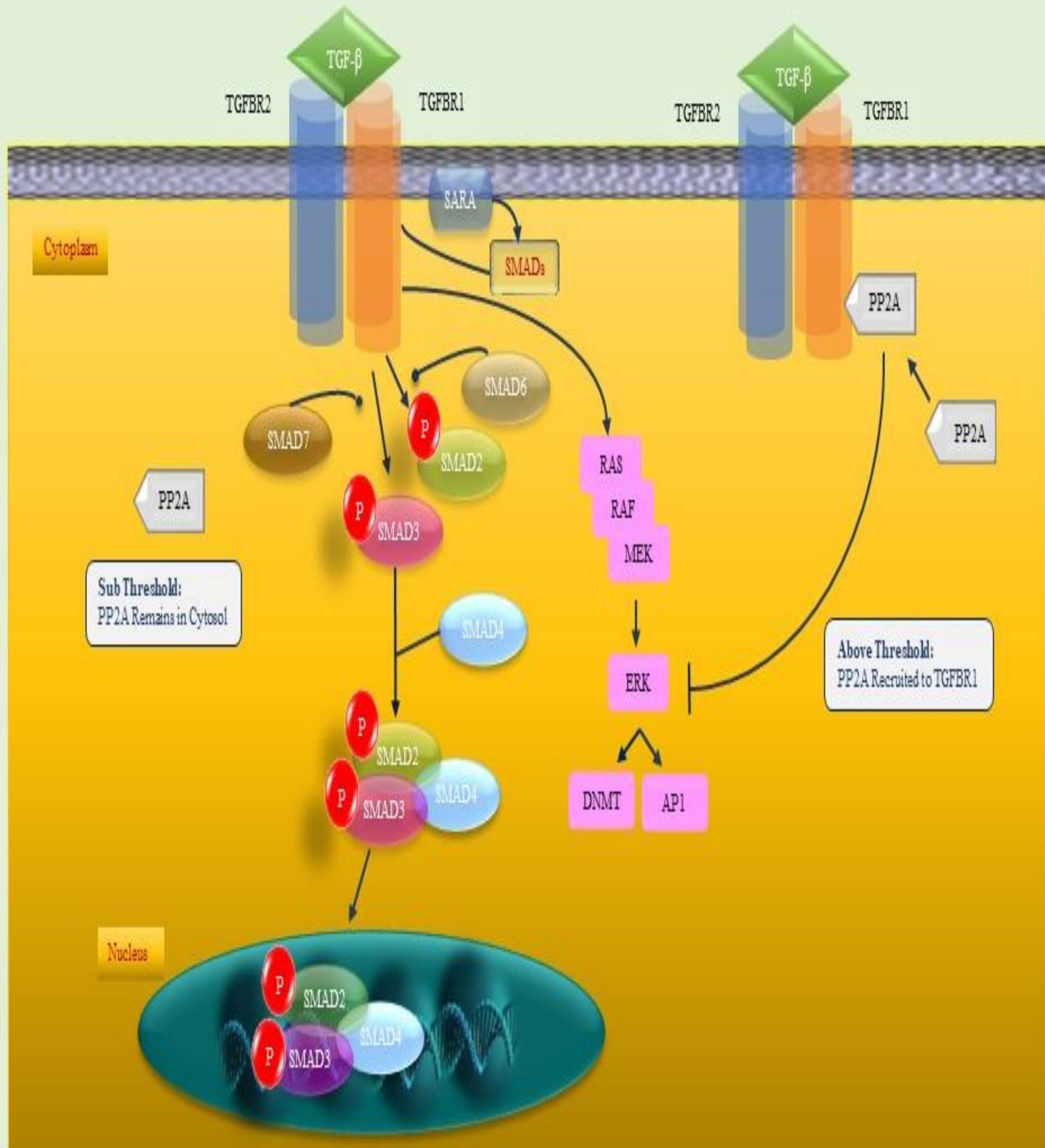

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Neutralization of TGF-β

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Transforming growth factor beta (TGF-β) regulates a broad range of cellular processes including cell proliferation, differentiation, homeostasis, and embryonic development. However, it plays a crucial role in the pathogenesis of many diseases including cancer, cardiovascular, and autoimmune diseases. The goal of this study is to identify new strategies for selective modulation of the overexpression of TGF-β signaling, which may provide a potential role in preventing the effects caused by this cytokine. To achieve this goal, this study sheds the light on how the mode of action of TGF-β is, and how the protein kinases influence their substrate proteins; how the relative activities between protein kinase and protein phosphatase behave, and how such interactions influence the conformation and activity of protein kinases. In addition, this study provides new therapeutic proposals, which might employ as a reference for the study of the neutralization of TGF-β.

Keywords: TGF-β, SMAD, Protein kinase, Protein phosphatase, PPM1A, Myristoylation, CSIG.

1. Introduction

Transforming growth factor beta (TGF-β) is a multifunctional regulatory protein that is expressed in mammalian development, differentiation, and homeostasis in almost all cell types and tissues. However, the overproduction of TGF-β is implicated in multiple tumor types,[1,2] and it is thought to play a pro-tumorigenic angiogenesis, metastasis, tumor cell epithelial to mesenchymal transition, fibroblast activation and desmoplasia through effects on the tumor microenvironment by enhancing tumor cell invasion and by inhibiting the function of immune cells.[3,4] Studies of TGF-β expression in epithelial cancers have shown that TGF-β has a paradox function that it can function as a tumor suppressor and a tumor promoter.[5-8] TGF-β can have tumor suppressive properties in early-stage disease,[5,9] while harboring a tumor-promoting effect in more advance stages. Additionally, TGF-β is implicated in many diseases including the pathogenesis of diabetic tubular hypertrophy and fibronectin-stimulatory effects of high glucose,[10] chronic respiratory diseases, skeletal diseases, and Loeys–Dietz syndrome (LDS) (an autosomal dominant genetic connective tissue disorder).[11]

The objective of this study is to identify strategies for selective modulation of the overexpression of TGF-β signaling pathways, which may provide a possible role in preventing the effects caused by TGF-β overexpression. To achieve this goal, it is important to understand how the TGF-β signaling mechanism is and how

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the convergence between TGF-β and other pathways behaves. Thus, I briefly shed the light on these topics, including the strategies for targeting TGF-β signaling.

2. TGF-β signaling pathways

TGF-β comprises three mammalian isoforms (TGF-β1, TGF-β2, and TGF-β3), which each of them is essential for the development and is initially synthesized as a 75-kDa homodimer known as pro-TGF-β. In Golgi, pro-TGF-β is cleaved to form the mature 25-kDa TGF-β homodimers, which they interact with latency-associated peptide (LAP), a protein derived from the N-terminal region of the TGF-β gene product, forming the small latent complex (SLC).[12,13] In the endoplasmic reticulum, a single latent TGF-β binding protein (LTBP) forms a disulfide bond with TGF-β homodimer to form the large latent complex (LLC), allowing for targeted export to the extracellular matrix, which further interacts with fibronectin fibrils and heparin sulfate proteoglycans on the cell membrane. Finally, the large latent complex localizes to fibrillin-rich microfibrils in the extracellular matrix containing both the LTBP and the LAP, and there it is stored and remains biologically unavailable until its activation.[12,14] There are two distinct mechanisms to explain how the TGF-β can be activated from LLC. The one mechanism is an integrin-dependent TGF-β activation.[15] Latent TGF-β complex contains arginine-glycine-aspartate (RGD) sequence in C-terminal end of the latency associated peptide (LAP) chain, which shows high affinity to αv integrins, and is required for interaction with integrin. Once integrin interacts with latent TGF-β complex via RGD sequence, mature TGF-β can be released (activated) by conformational change of the whole complex without a need for proteolytic digestion. Among a subset of the integrin family, which are transmembrane receptors involved in cell-cell and cell-matrix signaling pathways during fibrosis, is αvβ1 integrin. αvβ1 integrin is the principal integrin that binds to and activates latent TGF-β complex and this activity is mediated in tissue fibrosis. As an alternative mechanism, mature TGF-β can be released from the LLC through several factors, including proteases, thrombospondin 1, retinoic acid, fibroblast growth factor 2 (FGF2), and reactive oxygen species, which release mature TGF-β from the microfibril-bound large latent complex. This takes place through liberation from LAP, degradation of LTBP, or modification of latent complex conformation.

The activated TGF-β ligands transfer signals through type I and type II receptors (TGFBR1 and TGFBR2), which are transmembrane serine/threonine kinases that serve to propagate the signal intracellularly. TGFBR2 binds the ligand and then recruits TGFBR1 into a heterotetrameric complex. TGFBR1 is activated upon phosphorylation by TGFBR2 and then phosphorylates downstream effectors. The TGF-β receptor complex transduces signals through what are known as canonical and non-canonical pathways.[16-18] The canonical TGF-β signaling pathway uses transcription factors SMAD2 and/or SMAD3 to transfer signals. SMAD2/3 are directly phosphorylated in their C-terminal SXS motif by TGFBR1 then hetero-oligomerize with SMAD4, and translocate to the nucleus, where they cooperate with specific DNA binding transcription factors to regulate gene transcription in a context-dependent manner (Figure 1).[15] In addition to these two receptors, there is a type 3 TGF-β receptor (TGFBR3 or betaglycan), a transmembrane proteoglycan that binds the TFG-β ligand, whose function may have a role in development and regulating TGFBR1 and TGFBR2.

Among the non-canonical TGF-β signaling pathways, extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) are involved. ERK and other MAPKs regulate the SMAD-

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dependent arm of TGF-β signaling through phosphorylation of SMAD2 and SMAD3 at specific amino acid residues. The ERK/MAPK pathway is induced by various stimuli. Activated ERK1/2 phosphorylates many proteins, including transcription factors, to regulate target genes and differentiation.[19,20] On the other hand, TGF-β signals through a number of non-SMAD pathways, such as c-Src, m-TOR, RAS, protein phosphatase 2A (PP2A/p70s6K), and JNK MAPK. Both SMAD-dependent signaling and SMAD-independent signaling play multiple roles in homeostasis, particularly in the growth and plasticity of epithelial cells. SMAD-dependent TGF-β signaling induces growth arrest through a number of mechanisms, and control over various cyclin-dependent kinase inhibitors, whereas SMAD-independent mechanisms of TGF-β-induced apoptosis involved the adapter protein Daxx that facilitates JNK activation.[15]

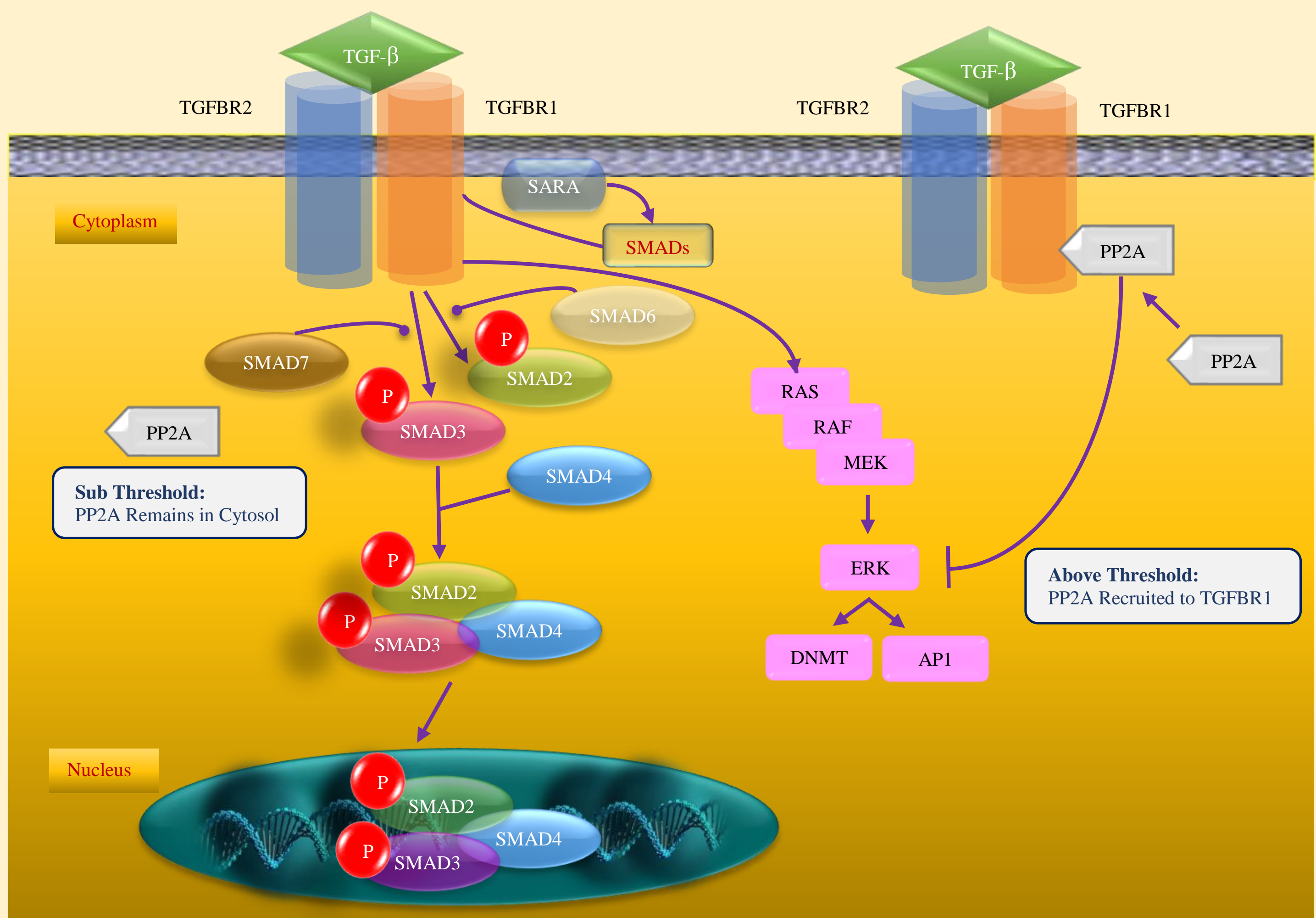

Figure 1. The TGFBR1 (orange receptor) and TGFBR2 (blue receptor), which are transmembrane serine/ threonine protein kinase (STKs) receptors, act as homodimers on the cell membrane. TGF-β ligand binds to TGFBR2, inducing its dimerization and enabling the TGFBR2 homodimer to form a stable hetero-tetrameric complex with the TGFBR1 homodimer. This process leads to subsequent

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activation of SMAD2 and SMAD3, and SMAD-independent pathways (including ERK). Hypothetically, in some cells, there is a reciprocal control mechanism involving ERK and TGFBRs. ERK activation leads to DNA methyltransferase (DNMT) and activator protein (AP1) expression, which is believed to lead to epigenetic repression of the TGFBRs and upregulation of the TGF-β ligand. Additionally, TGF-β suppresses ERK activation through a process involved recruitment of protein phosphatase 2A (PP2A) to the cell membrane by TGFBR1. At high level of PP2A is recruited, it serves as a barrier to ERK activation, where DNMT and AP1 expression are not induced, and the canonical SMAD arm of the TGF-β cascade is favored.[15] P = phosphorylation.

3. Phosphorylation of SMADs

SMADs are classified as canonical effector proteins, including receptor-regulated SMADs (R-SMADs; SMAD2/3); the common partner SMADs (co-SMAD; SMAD4) acting as transcription factors that regulate the expression of certain genes; and inhibitory SMADs (I-SMADs; SMAD6/7) that function as negatively regulate R-SMAD activation and nuclear translocation (Figure 2).

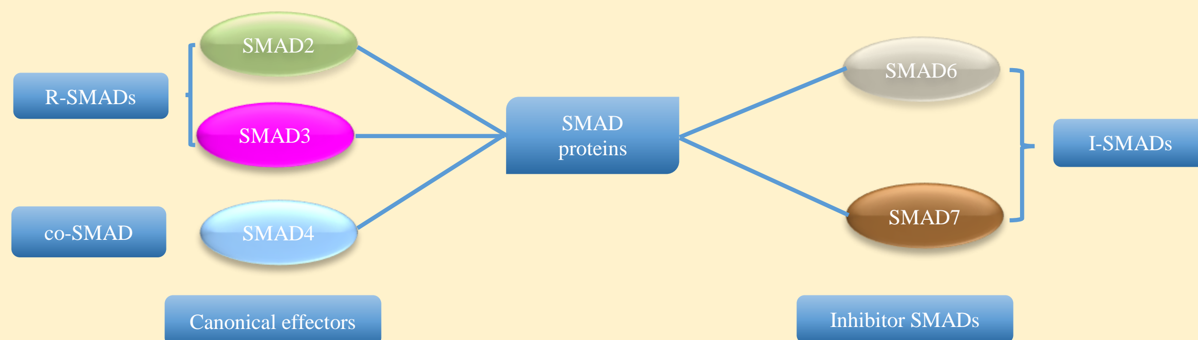

Figure 2. Schematic classification of SMAD proteins. SMADs are small intracellular effector proteins characterized by homologous regions at their N- and C-termini, known as MAD homology domains MH-1 and MH-2 respectively. These domains are connected by an intermediate linker, which recruits ubiquitin ligases and is phosphorylated by other signaling kinases such as MAPKs and cyclin dependent kinase (CDKs).

SMADs are essential intracellular transducers for TGF-β signals. Signal transduction pathways are often regulated by dynamic interplay between protein kinases and phosphatases. TGF-β ligand stimulation results in R-SMAD phosphorylation, inhibition of nuclear export, and thus persistent accumulation of SMAD complexes in the nucleus.

4. Protein phosphatases

The level of phosphorylation of any protein depends on the relative activities of protein kinases (PKs) and protein phosphates (PPs). Proteins can be phosphorylated on either serine, threonine, or tyrosine residues. Whereas protein serine/threonine phosphatases (PS/TPs), a form of phosphoprotein phosphatase that acts

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upon phosphorylated serine/threonine residues, control cellular functions through cleavage of phosphate from phosphorylated serine and threonine residues in proteins (Figure 3). In fact, PPs are not just passive counterparts of PKs, but are regulated in a sophisticated manner and play an active and essential role in the regulation of cellular processes. Among the three structurally distinct families of PS/TPs is the PPM family, which comprises metal-ion-dependent phosphatases, and includes PPM1A. PPM1A has magnesium/ manganese ion dependency, and possesses a catalytic subunit that can be located in the nucleus or the cytoplasm. PPM1A can specifically bind to its substrates and catalyze the dephosphorylation of these substrates. Thus, PPM1A dephosphorylates the critical C-terminal SXS motif of SMAD2/3 resulting in disassembly of the transcriptionally active R-SMAD/Co-SMAD, initiating a molecular cascade for termination of the transcriptional SMAD signal, and, hence, promotes nuclear export of SMAD2/3 to cytoplasm leading to terminate TGF-β signaling.

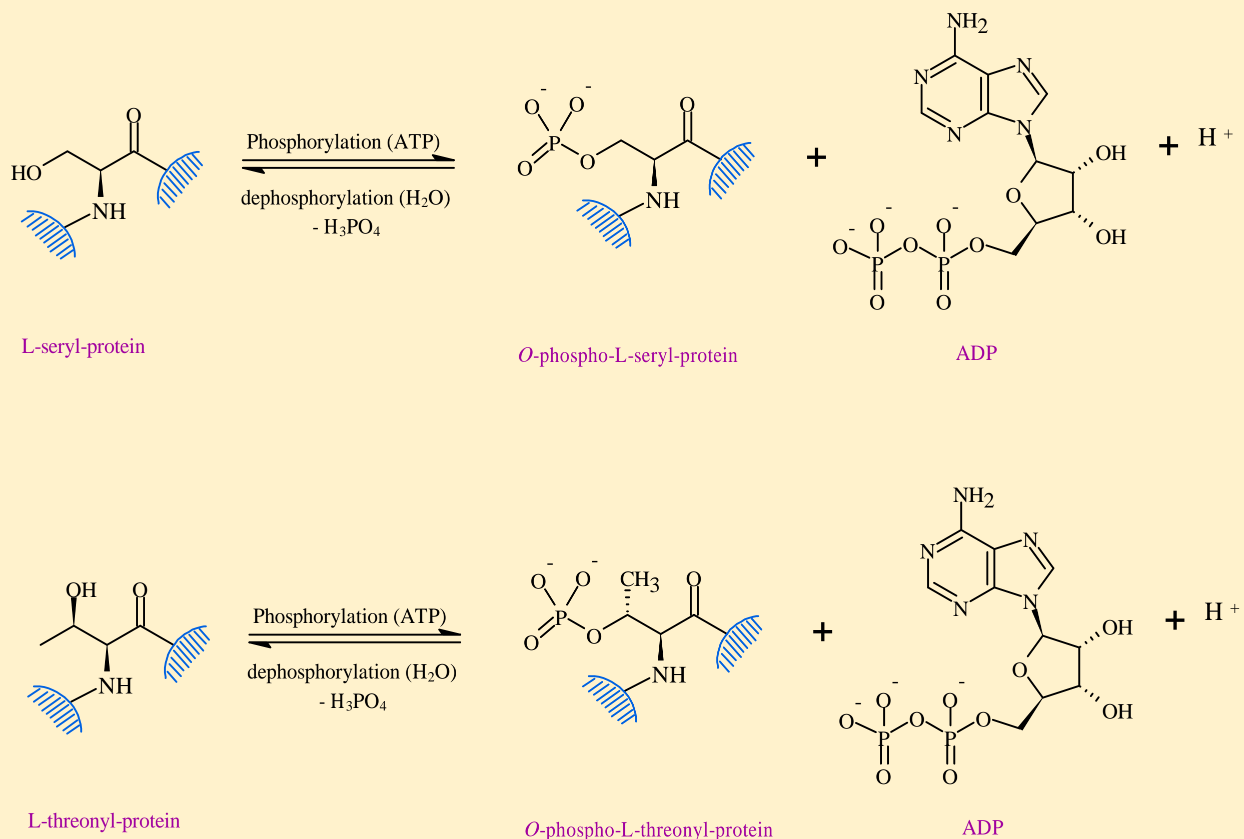

Figure 3. The phosphorylation and dephosphorylation of L-serine and L-threonine residues of the protein.

In response to TGF-β signaling events, SMAD7 is recruited to TGFBR1, blocking SMAD2/3 phosphorylation and its downstream signaling, as well as indirectly repress TGF-β signaling by targeting its receptors (TGFBR1, TGFBR2), and other SMAD transcription factors for proteasomal degradation.

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Taken together, the PPM1A protein participates in signal transduction in the TGF- signaling pathway by dephosphorylating the phosphorylated SMAD2/3 serving as an important countermechanism for terminating SMAD signaling. Therefore, the enzymatic activity of PPM1A can influence the regulatory effect of the TGF-β signaling pathway through dissociation of the SMAD complex, and subsequent nuclear export of SMAD2/3. [21] Increasing studies indicate that PPM1A can be modified by myristoylation, a process that is essential for PPM1A to achieve its role of dephosphorylation its physiological substrates in cells. [22]

3.1. N-Myristoylation

Myristoylation shares palmitoylation, prenylation, and glycosylphosphatidylinositol (GPI) anchoring as the major types of protein lipidation. Myristoylation influences the biofunction of proteins, including the charge, hydrophobicity, and other aspects of targeted-protein chemistry, leading to pronounced variations in the physiology of the targeted protein, such as conformation, trafficking, localization, and binding affinity for cofactors (Figure 4). N-myristoylation involves the addition of myristate, a 14-carbon fatty acid, to the N-terminal glycine residue of a protein via a covalent amide bond. Rarely, myristic acid ($C_{13}H_{27}COOH$) is attached to lysine residue, a process known as lysine myristoylation. N-myristoylation on a protein involves irreversible covalent bonding, pointing out that the myristoyl motif may orient the protein toward a specific destiny. However, this irreversibility cannot shield the myristoylated protein from interference with other proteins to regulate protein functions. The N-myristoylation mechanism catalyzed by N-myristoyltransferase (NMT) behaves via two pathways: i) Cotranslational modification with the most accurate steps occurring after the removal of the methionine initiator by methionine aminopeptidase (MetAP); and ii) Posttranslational modification on an internal glycine exposed by caspase cleavage during apoptosis (Figure 5). [23]

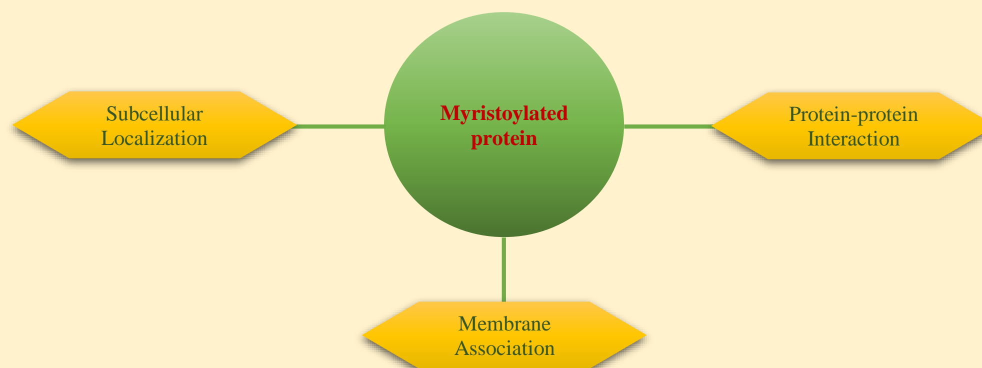

Figure 4. Schematic biofunction of myristoylation.

The process of myristate transfer is achieved by NMT during the multiple enzymatic steps of N-myristoylation. There are two isozymes of NMT, referred to as NMT1 and NMT2, which differ from each other in the N-termini, and are encoded by distinct genes that exhibit different physiologic role of each isozyme.

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A myristoyl group, as a hydrophobic motif, facilitates protein binding in membranes leading to conformation changes and improving the hydrophobicity of the protein. In addition, it affects protein localization and the ease with which a protein binds to substrates. N-myristoylation is an evolutionarily conserved lipidation that is essential for cell viability in various organisms, including eukaryotic cells and viruses. However, some N-myristoylated proteins in small RNA viruses and retroviruses are essential for virus assembly during viral replication or production of infectious viral particles, which may be involved in the survival and propagation of pathogens. Furthermore, some pathogens need to utilize host cellular machinery to replicate within host cells due to deficiency in viral NMTs. Therefore, different approaches have been revealed that therapeutic interventions of protein N-myristoylation through NMT inhibitors can be considered as targets for diseases caused by proliferating cells or pathogens, such as infectious diseases caused by various pathogens and malignancies.[23]

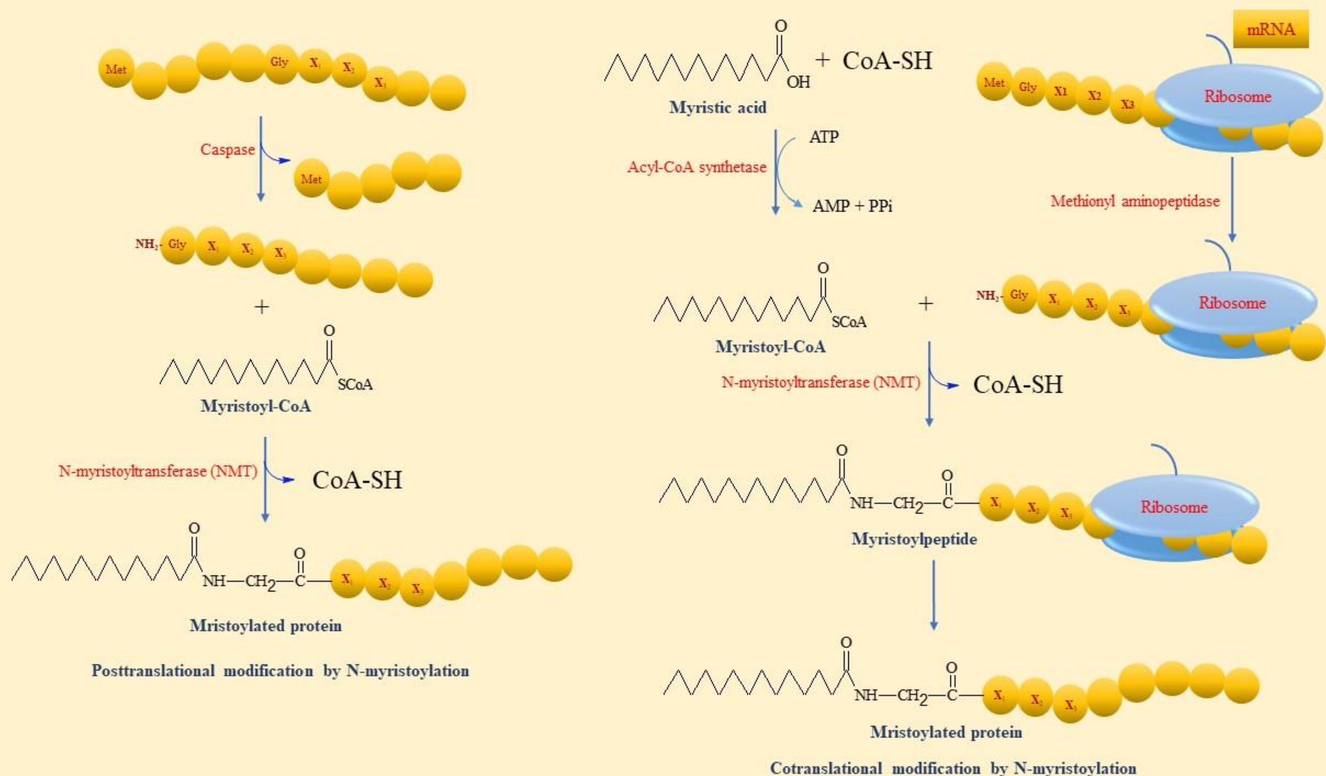

Figure 5. Schematic pattern of the N-myristoylation mechanism catalyzed by NMTs.[23] CoA-SH: coenzyme A, ● : amino acids.

3.2. *The cellular senescence-inhibited gene (CSIG)*

A previous research has revealed that the cellular senescence-inhibited gene (CSIG) serves as a regulator of PPM1A myristoylation and TGF-β signaling. CSIG is a nucleolar protein, which its N terminus comprises a

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ribosomal L1 domain, while its C terminus is attached to a lysine-rich domain. CSIG is involved in different biological processes, such as cellular senescence and apoptosis. It has shown that CSIG knockdown reduces PPM1A myristoylation and subsequently inhibits the dephosphorylation of phosphorylated SMAD2. CSIG promotes the binding of PPM1A to SMAD2, and is essential for the combination between PPM1A and NMT1.

In fact, PPM1A translocates from its original location in the nucleus to the cytoplasm and that TGF-β signaling is enhanced upon CSIG knockdown.[24] By promoting the myristoylation of PPM1A, CSIG enhanced the dephosphatase activity of PPM1A and further inhibited TGF- signaling (Figure 6).

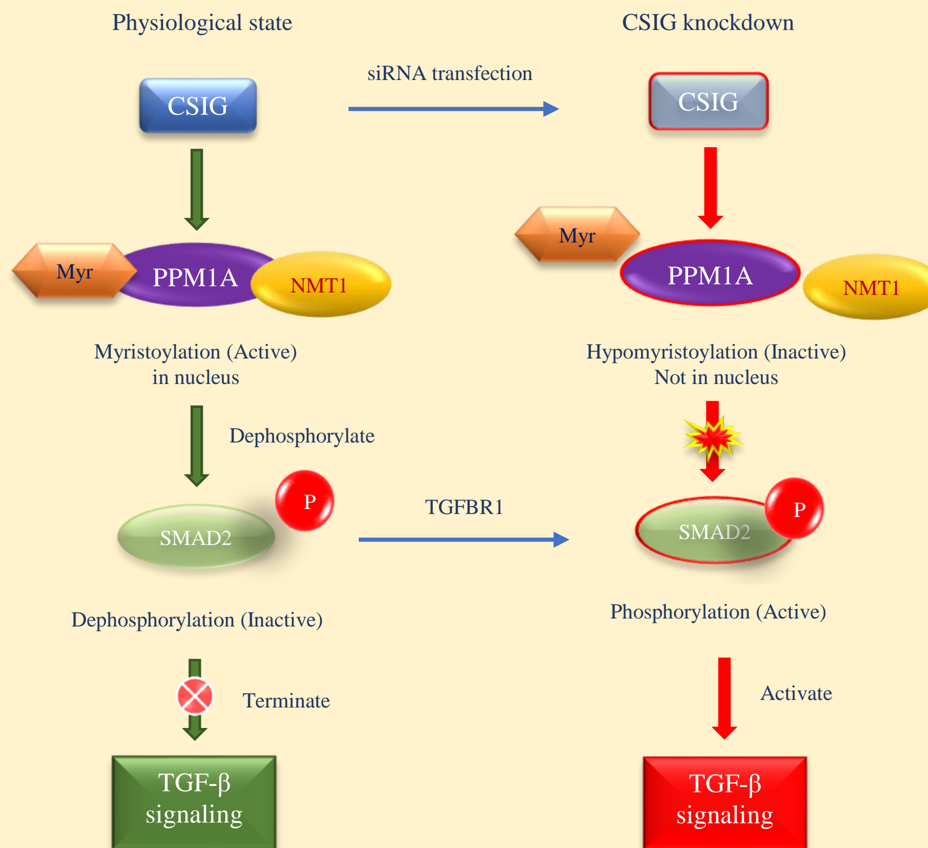

Figure 6. Systematic diagrams explaining the regulatory role of CSIG under normal physiological and knockdown conditions. Under normal conditions, PPM1A exhibits high phosphatase activity as a result of excess level of CSIG expression, and, hence, dephosphorylates the phosphorylated SMAD2 terminating the TGF-β signaling (left). While after CSIG knockdown by siRNA transfection, the phosphatase activity of PPM1A decreases sharply due to inhibiting of PPM1A myristoylation resulting in increased levels of the phosphorylated SMAD2, and the enhancement of TGF-β signal transduction (right).[24] Myr: myristoylation; NMT1: N-myristoyltransferase 1; P: phosphorylation.

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4. The convergence between TGF-β and other pathways

TGF-β1 is a pleiotropic cytokine which can mediate a wide spectrum of cellular effects through a variety of signaling pathways. It is a well-known feature that sometimes different pathways cooperate to orchestrate a certain cellular effect. Some of these pathways will be further discussed demonstrating the behavior of TGF-β in the tumor environment.

TGF-β is tightly regulated by MAPK, that is associated with receptor tyrosine kinases. TGFBR1 has tyrosine kinase activity, which phosphorylates tyrosine on ShcA (a Src homology and collagen A adaptor protein that binds to tyrosine kinase receptors) leading to Ras activation and downstream ERK phosphorylation.[25] Enhancement of SMAD-dependent arm of TGF-β signaling by ERK and MAPK through phosphorylation of SMAD2 and SMAD3 may be associated with the abnormal behavior of TGF-β in cancer, as demonstrated by SMAD3 phosphorylation at Ser208/213 residues manifested in advanced tumors.[26]

TGF-β acts as a self-regulator of its expression, and changes in its regulation can lead to downregulation or inactivation of the TGFBRs at both the epigenetic and protein levels. Such receptors repression may be accompanied by a compensatory induction of the TGF-β ligand, which may result in a cell-autonomous tumor promotor through non-canonical pathways as the case in some cancers.[27]

TGF-β constitutes an important regulator of gene methylation, a process dependent on ERK activation of DNA methyl-transferase (DNMT). DNA hypermethylation affected TGFBR1 and TGFBR2 genes, and downregulating their expression.[28] In some cases, TGF-β suppresses ERK activation through a process that involves protein phosphates 2A (PP2A). PP2A is synthesized in the cytoplasm and can be recruited by activated TGFBR1 to the cell membrane depending on the level of TGF-β available to the target cells. It can serve as a regulator of TGF-β receptor stability and activity[29], as well as a context-specific inhibitor to ERK activation. In response to high level of TGF-β, a sufficient quantity of PP2A is recruited by activated TGFBR1 serving as a barrier to ERK activation, where DNA methyltransferase (DNMT) and activator protein (AP1) expression are not induced, and the canonical SMAD arm of the TGF-β cascade is favored. Whereas, at a low level of TGF-β, the quantity of PP2A available is restricted due to the limited quantity of activated TGFBR1, and ERK remains activated (Figure 1).[15]

In fact, TGF-β contributes in tumor progression. TGF-β is implicated in sustained angiogenesis, a critical process in tumor progression, facilitating nutrient exchange and metastasis.[30] Tumor-derived TGF-β affects proximate stromal and endothelial cells (ECs), as well as vascular endothelial growth factor (VEGF) expression.

In tumor microenvironment, TGF-β plays a key role in the repression and evasion of immune surveillance. TGF-β can suppress or alter the activation, maturation and differentiation of both innate and adaptive immune cells, including natural killer (NK) cells, dendritic cells (DCs), macrophages, neutrophils, and $CD4^+$ and $CD8^+$ T cells.[4] In terms of the innate immune response, TGF-β inhibits IFNγ production by NK cells causing dampened $CD4^+$ T_H1 cell responses. TGF-β inhibits DC maturation and cytokine production, thereby promoting a tolerogenic environment. Moreover, TGF-β produced by tolerogenic DCs contributes to T_{reg} cell differentiation. TGF-β can also favor the differentiation of M2 versus M1 phenotype of macrophages by

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inhibiting NF-κB activation. Besides, TGF-β promotes the differentiation of N1 to N2 neutrophils, which similar to M2 macrophages, are less cytotoxic. In terms of the adaptive immune response, TGF-β can also directly dampen the function of $CD8^+$ and $CD4^+$ T cells while promoting the recruitment and differentiation of regulatory T cells (T_{regs}) at the tumor microenvironment. TGF-β also controls the differentiation of several key $CD4^+$ T cell subsets in tumor immunology, including T_H1, T_H17 and T_{reg} cell subpopulations. The impact of TGF-β on the differentiation of $CD4^+$ T cells is influenced by the cytokine milieu in the tumor microenvironment. In addition to impairing T-cell effector function, TGF-β has been considered as a powerful inducer of T_{regs}, which their development and function are orchestrated by the forkhead family transcription factor (Foxp3). Strikingly, TGF-β signaling in T cells participates in the expression and enrichment of Foxp3. This may be relevant to the fact that high level of $CD4^+CD25^+Foxp3^+$ T_{regs} in tumors is represented clinically a marker for identifying high-risk breast cancer patients.[31,32]

In fact, TGF-β plays a pivotal role in tumor evasion of the immune system in cancer, which renders advanced tumors progressively less recognizable to the surveillance program. In other words, TGF-β provides the tumor with a cloaking mechanism, facilitating evasion of immune system surveillance.[33,34] So, blocking TGF-β can induce an expression profile in the tumor microenvironment that promotes better antigen uptake and presentation, resulting in more robust priming and activation of the adaptive anti-tumor immune response.

5. The current therapeutic strategies targeting TGF-β signaling

In view of the switch function of TGF-β, a wide array of approaches has been developed to neutralize TGF-β signaling ranging from monoclonal neutralizing antibodies targeting TGF-β proteins that bind to ligands and prevent access to TGF-β receptors[35] to the use of soluble forms of TGFBR3 (betaglycan) to sequester TGF-β from the cellular receptors.[36] In fact, the current therapeutic strategies against TGF-β can be classified into three levels: i) the ligand level through prevention of TGF-β synthesis by using antisense molecules; ii) the ligand-receptor interaction level, which prevention of the interaction takes place by ligand traps and anti-receptor monoclonal antibodies; and iii) the intracellular level through prevention of signal transduction by receptor kinase inhibitors and peptide aptamers.[37] Many of these approaches have yielded highly promising results. However, the high unmet need in effective immunosuppressive treatment calls for the development of new therapy methods. Here I will introduce some possible scenarios which may inhibit the overexpression of TGF-β, and may result in a potential promise of providing solutions to the challenges of neutralizing TGF-β.

6. Hypothetical scenarios targeting TGF-β signaling

In the light of the above discussion, one can envisage that among the various strategies for neutralizing TGF-β signaling are: i) regulating the conformation of protein kinases to enhance its stability and regulatory process toward other proteins; ii) inhibiting the phosphorylation process of SMAD2/3, where the hyper-phosphorylation of SMADs leads to the overproduction of TGF-β, and hence one of the signatures of many diseases occurrence; and iii) blocking the LAP activation through the dissociation of the disulfide linked homodimer of the LAP peptides. Such intracellular strategies may effectively address the challenge of TGF-β

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overproduction. TGF-β is a pleiotropic cytokine and a major pro-fibrogenic cytokine associated with many diseases, thus, therapies targeting this cytokine require a synergistic agent with combined activities to produce a joint effect to regulate the dual nature of TGF-β signaling.

The most important central problem in regulating of TGF-β is a consideration of stereochemistry. In other words, the correct conformation of protein kinases (the serine/threonine and tyrosine kinases) have a dominant influence in TGF-β regulation. Uncontrollable spatial and temporal phosphorylation of specific serine, threonine, or tyrosine residues leads to devastating consequences. Thus, it is important to shed the light on the structure and the catalytically active conformation of the protein kinases in short.

6.1. The conformation of protein kinases

The proper regulation of protein kinases depends on the spatial and temporal control of phosphorylation of their residues. These enzymes are molecular switches that can adopt two extreme conformations: an "on" state that is active, and an "off" state that is inactive. The catalytic mechanisms of these protein kinases are involved the transfer of the γ-phosphate of ATP to the hydroxyl group of serine, threonine, or tyrosine. Upon activation, they all adopt "on" conformation, that are structurally similar. Whereas their "off" conformations exhibit a remarkable plasticity, that allows the adoption of distinct conformations in response to protein-protein interaction, *i.e.* the adoption of the catalytically active conformation is hindered in different ways.

6.1.1. The structure in the 'On"state

The protein kinase fold is separated into two lobes: the smaller N-lobe, connected by a short hinge region to the larger C-lobe. N-lobe is composed of five-stranded β sheet and one α helix known as helix αC. Whereas C lobe is predominantly helical. ATP is bound in a deep cleft between the two lobes, and sits beneath a highly conserved loop connecting strands β1 and β2. This phosphate binding loop (P-loop) contains a conserved glycine-rich sequence motif. The glycine residues allow the loop to approach the phosphates of ATP very closely and to coordinate them through backbone interactions. The conserved aromatic site chain caps the site of phosphate transfer. In the absence of ATP, the glycine residues make the P loop very flexible, which facilitates the binding of small molecule inhibitors.[38] Some of these inhibitors induce large structural distortions in the loop by interacting with the conserved aromatic residues.
A centrally located loop known as "activation loop" provides a platform for peptide substrate, that binds in an extended conformation across the front end of the nucleotide binding pocket, close to the γ-phosphate of ATP. In the most kinases, this loop is phosphorylated when the kinase is active. Phosphorylation of the activation loop stabilizes it in an open and extend conformation that is permissive for substrate binding. Taken together, there are two key regulatory elements within the kinase domain, the activation loop and the αC helix.

6.1.2. The activation loop

The activation loop has the ability to undergo large conformational changes when the kinase switches between "off" and "on" states. In the unphosphorylation state, the activation loop collapses into the active site, block-ing the binding of both nucleotide and peptide substrate. Upon phosphorylation, it moves away from the

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catalytic center and adopts a conformation that allows substrate binding and catalysis. For example, the regulation of MAPK by phosphorylation of the activation segment shows that MAPK have a C-terminal extension that binds to the surface of the N lobe, near helix αC. Phosphorylation at Thr183 in the activation loop induces a conformational change in this C-terminal extension, exposing a hydrophobic surface that facilitates the formation of homodimers, a requirement for the nuclear localization of the enzyme.[38]

6.1.3. The αC helix

Helix αC constitutes a key mediator of conformational changes that take place within the catalytic center. It is the only conserved helix in the β-sheet rich N lobe, and located within it an absolutely conserved glutamate residue (Glu91 in PKA). In active kinases, this glutamate residue forms an ion pair with the lysine side chain (Lys72 in PKA) that coordinates the α and β phosphates of ATP. Additionally, the αC helix makes direct contact with the N-terminal region of the activation loop, and its conformation is linked to that of the conserved Asp-Phe-Gly motif. This interaction, along with the Glu91-Lys72 ion pair, directly couples the conformation of the helix to nucleotide binding. Regulatory mechanisms often adjust kinase activity by altering the conformation of helix αC, thereby affecting the integrity of these interactions.[38]

In fact, there is a structural coupling between the activation loop and αC helix; a switch in one necessitates a switch in the other. Such coupling allows for allosteric information to pass between the active site and the back of the enzyme, a general phenomenon in kinase regulation.

6.2. Autoinhibition and substrate recruitment by N-terminal segments in TGFBR1

Some groups of kinases are regulated by N-terminal segments that do not impinge upon the active site directly, but rather inhibit catalytic activity via conformation change alone. The TGFBR1 receptor is activated by phosphorylation of an N-terminal juxtamembrane segment of this receptor, known as the GS region. This segment contains 3-4 of the serine and threonine within it, which they must be phosphorylated in order to fully activate the enzyme. The unphosphorylated juxtamembrane segment of TGFBR1 receptor (the GS region) maintains the N lobe of the kinase in a distorted inactive conformation when this region is bound by the inhibitory protein FKBP12. Such conformational changes disrupt productive ATP binding. In other word, phosphorylation of the GS region disrupts the interaction with FKBP12 and the GS loop becomes flexible, and hence creates binding sites for substrate proteins of the SMAD family.[38]

6.3. Proposals for therapeutic intervention

Study of the above paradigm provides an insight into structure and function of protein kinase. To influence the overproduction of TGF-β, there is a need to introduce an auxiliary (molecule, peptide, or metal ion) to block the active site of kinase residues, or to stabilize the fold’s conformation of the protein by removing a specific group or atom.

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Small molecules such as diisopropylphosphorofluoridate (DFP) can be served as an active site inhibitor through blocking the active site of serine kinase (Figure 7). However, such small molecules suffer from specificity, and they might react with other reactive moieties on the protein surface.

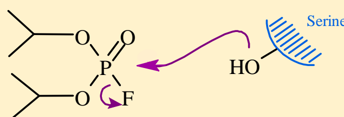

Figure 6. Blocking the active serine residue of the protein by diisopropylphosphorofluoridate.

The coordination of metal cations with the active site may imply the stability of the "on" conformation of the kinase. Furthermore, the cations may involve in coordinating some of the ligands that TGF-β bind, leading to disturb the local hydrogen bonding and the hydrophobic interactions, as well as the conformational dynamics of the region, which may generate a lower energy pathway of the transition state of the covalent intermediate to favor the active conformation. Such cations may play an active regulatory role, acting as physiological activators or inhibitors, that finely tune the conformational distribution. Metal cations such as Mn^{2+}, Mg^{2+}, Zn^{+2}, Co^{2+}, Ca^{2+} can be efficiently used as active site-directed inhibitors of the protein. Worthy to mention that, the PKA binds to two divalent metal cations (Mg^{2+}) in the active site during catalysis, implying the stability of the "on" conformation.

I have previously reported conceptual approaches to inhibit the overexpression of phospholipase D (PLD) signaling and its product phosphatidic acid (PtdOH), which are associated with enhanced tumorigenesis.[39] These possible scenarios include halopemide compounds; butylated hydroxyltoluene; zinc ions; pyridine carboxaldoxime-Zn(II) complex; and *cis*-β-hydroxoaquatriethylenetetramine cobalt(III). Among them, the metal complexes can probably be effective candidates for inhibiting phosphorylation processes. This related to their directional effect through polarization of the substrate and external attack, or ionization of particular reactive reagent through the concentration of a potent nucleophile at a biologically pH. In this context, I provide a rational explanation of using these metal chelate compounds as small molecule inhibitors targeting different functions of TGF-β signaling.

6.3.1. *cis*-β-hydroxoaquatriethylenetetramine cobalt(III)

Mimicking the affinity of αv integrins to the arginine-glycine-aspartate (RGD) motif of the TGF-β latency-associated peptide (LAP) chain, through blocking the latent TGF-β complex, may probably be an effective pathway in targeting the TGF-β signaling. I speculate that upon complexation of the complex ion catalyst *cis*-β-hydroxoaquatriethylenetetramine cobalt(III), $[Co(trienH_2O)OH]^{2+}$, with RGD sequence, the conformational change of the latent TGF-β complex may probably be restricted as a result of the hydrolyzed product remaining firmly bound to the metal center on one hand, and, a favorable decreasing in $\Delta S^{\ddagger}$ (while $\Delta H^{\ddagger}$ remaining unchanged) as a characterized factor of such process on the other hand. The outcome of such complexation would probably block the interaction between the αv integrin molecules and the latent TGF-β

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complex, thereby preventing the mature TGF-β from being released. The catalytic reaction of RGD hydrolysis can presumably be outlined as that the initial step may involve the replacement of the coordinated water molecule by the NH_2- of guanidino group or NH_2- of α-amino group of arginine followed by the carbonyl coordination, then the liberation of the peptide minus the arginine residue. The reaction can be postulated in (Figure 7). However, the carboxylate groups of aspartic acid might be implicated in the hydrolysis by contributing to the stability of the complex.

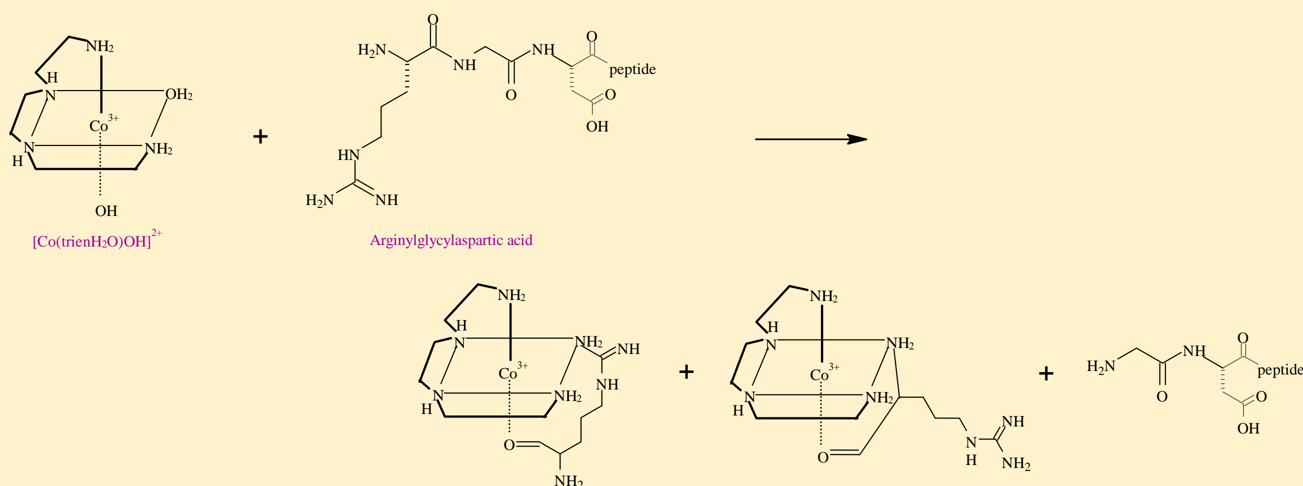

Figure 7. Proposed mimicking the affinity of latency associated peptide (LAP) chain to integrin family through the dissociation of arginine-glycine-aspartate (RGD) sequence in the C-terminal end of LAP.

6.3.2. Selenols

Another scenario is dissociation of the disulfide linked homodimer of the LAP peptides, which are assembled after proteolytic process as a non-covalent complex of a disulfide linked homodimer of the mature TGF-β dimer and a disulfide linked homodimer of LAP (the small latent complex). As a potential catalyst for the dissociation of disulfide bond in biological environment is selenols, which acts as a strong nucleophile toward disulfide yielding selenosulfide (Figure 8). The catalytic activity of selenols is related to a combination of the low pK_a (ca. 5.5 to 7), and hence significantly high concentration of RSe^- at pH 7 for selenols, and weak solvation and high polarizability, and hence high nucleophilicity of the selenolate anion.[40] Both of selenols and diselenides can be conveniently used to catalyze reactions involving strongly reducing dithiols.

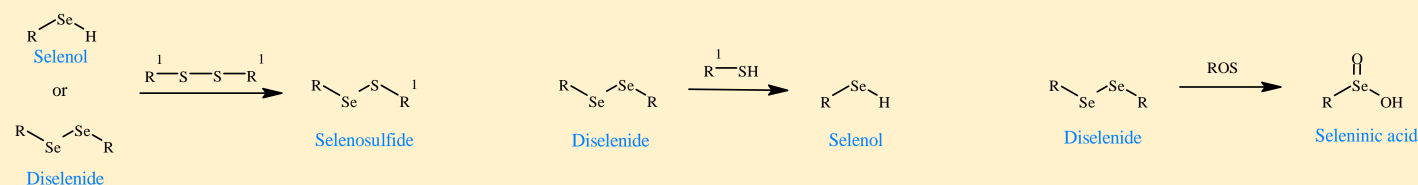

Figure 8. The reaction of selenol and diselenide with disulfide; thiol; and reactive oxygen species (ROS).

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In fact, selenium has the ability to regulate the redox balance in the mammalian, and serves as the active center of antioxidant enzymes such as glutathione (GSH) peroxidase (GPx), thioredoxin reductase (TrxR), and iodothyronine deiodinase (DIO). Furthermore, selenium-containing compounds are reported to show both antioxidant and pro-oxidant abilities in different concentrations and environments. Selenium-containing nanomaterials are used as drug delivery carriers to achieve the controlled release of anticancer drugs, because of their sensitivity to certain stimuli, such as redox, light, and radiation. Additionally, some types of selenium-containing nanomaterials possess anticancer activity on their own.[41] Mechanistic studies demonstrate that selenium switches between antioxidant properties at low nutritional levels and pro-oxidant at elevated doses, which induces the production of reactive oxygen species (ROS) by consuming GSH in cancer cells. Because cancer cells are more vulnerable to additional ROS production, the coordination of selenium-containing nanomaterials with ROS induction properties demonstrate the selectivity of higher toxicity in cancer cells than in normal cells.[42,43]

The redox responsiveness diselenide- or monoselenide containing hyperbranched polymers exhibited antiproliferation properties against different kinds of cancer cells by inducing cell apoptosis.[44] Furthermore, selenium nanoparticles (SeNPs) can interact with gold,[45] where the selenium-prepared AuNPs showed the inhibition of the invasion and metastasis by downregulating the matrix metalloprotein-2 expression in cancer cells.[46] Selenium could also coordinate with divalent cations such as Zn^{2+}, and Cu^{2+}.

6.3.3. Divalent metal cations

Divalent metal cations are excellent electrophilic catalysts in the chemistry of phosphates. The hydrolysis of ATP is susceptible to catalytic action of a variety of metal ions (M^{2+}) such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, which may inhibit the phosphorylation process of SMADs, and protein kinase residues. As a proposed approach, 2-hydroxymethyl phenanthroline-M^{2+} may stimulate ATP hydrolysis (Figure 9). Such complex compounds may also perform such a role in de phosphorylation of SMADs.

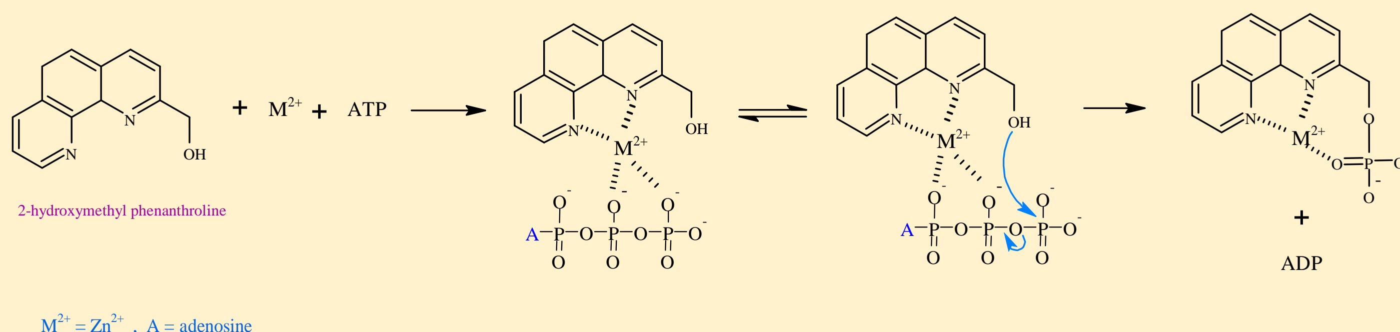

Figure 9. Metal complex compounds mediate ATP hydrolysis.

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6.3.4. Multifunctional hybrid nanoparticles

TGF-β is a complex cytokine that employs more than one regulating parameter during the course of its action. Thus, in search of a multifaced therapeutic agent targeting TGF-β signaling, I propose an aptamer-functionalized Au@MnO nanoflowers as a novel conceptual approach.

Multifunctional hybrid nanoparticles have characterized by their unique properties such as multipurpose use, prepared easily in different shapes, and high surface area that can carry more than one functional group without significant interferences between them due the presence of different discrete domains. A nanocomposite containing gold (Au) and manganese oxide (MnO) domains provides two functional surfaces for the attachment of different molecules, hence, increasing the therapeutic potential. I previously reported, as a hypothetical concept, the aptamer-functionalized Au@MnO nanoflowers that may serve as a therapeutic option for CVID-19.[47] Thus, I speculate that employing a multifunctional hybrid nanoparticle incorporating aptamers may produce a collective effect in targeting TGF-β.

Aptamers have the ability to bind with high affinity to target proteins, which, in turn, inhibit the binding to their receptors. In addition, they are able to recognize epitops and efficiently penetrate into biological compartments.[47] They may selectively recognize either an active or diseased-associated protein conformational state. Moreover, they have the possibility of chemical modification and rapid folding properties. Aptamers can be selected from combinatorial libraries on the basis of their affinity and specificity to the target protein. For instance, yeast two hybrid based approaches have been extensively used for identifying aptamers able to bind the proteins of interest.[48,49]

It is well known that TGF-β latency-associated peptide (LAP) function as endogenous ligands for αv integrin molecules. Therefore, targeting αv integrin through inhibiting integrin-mediated cellular functions, in particular latent TGF-β activation, may be an effective therapeutic option. Therefore, selection of integrin blocking aptamers that have the affinity and specificity to αv integrin molecules, in particular αvβ1 integrin, may contribute substantially to the inhibition of integrin-mediated latent TGF-β activation as well as its downstream signaling.

To protect the proposed integrin aptamer from nuclease degradation and to improve its stability *in vivo*, it is preferred to modify it with a hydrophilic poly(ethylene glycol) (PEG) block. For example, sbC-PEGylated aptamer was synthesized by Haruta *et al.* via coupling the symmetrical branching molecule 2-cyanoethyl-*N,N*-diisopropyl phosphoroamidite to the 5´ end of the aptamer, before conjugating two polyethylene glycol (PEG) molecules to the aptamer (Figure 10).[50] This modified aptamer can be anchored to the metal oxide petals.

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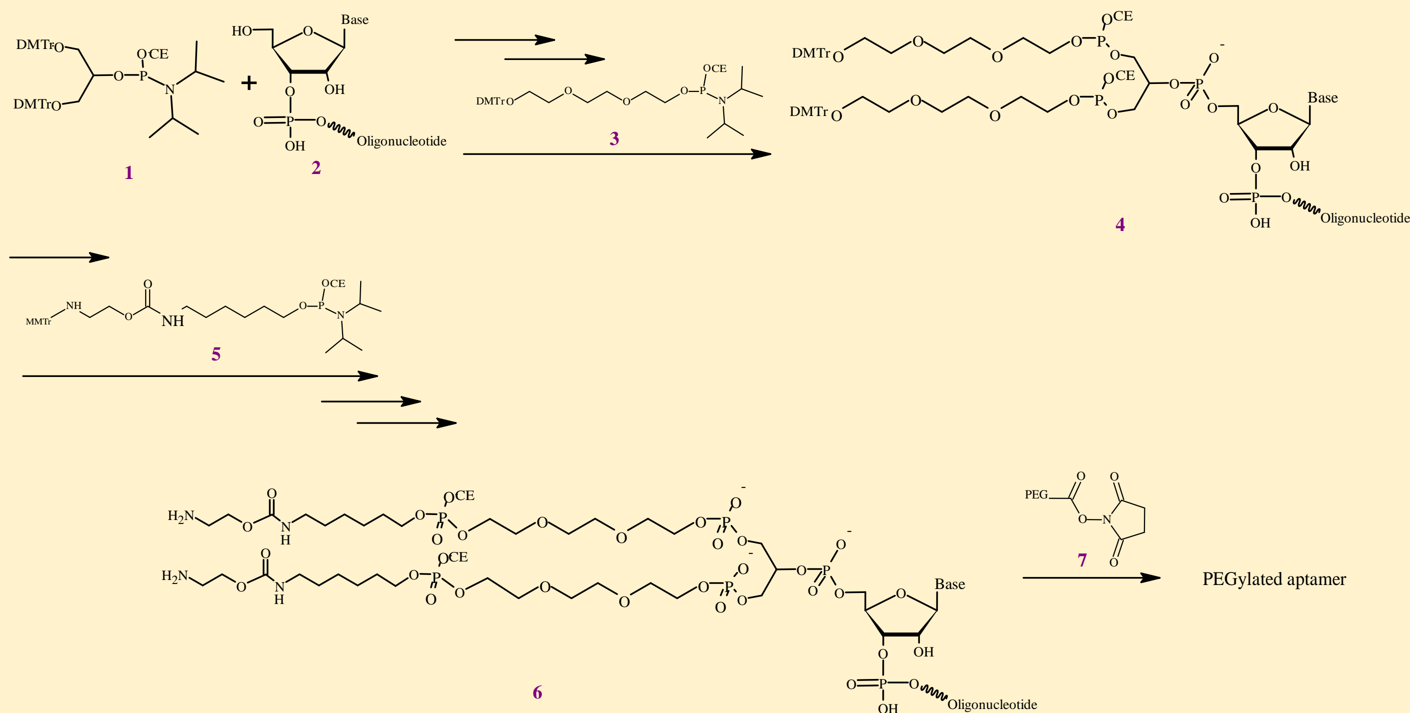

Figure 10. Preparation of modified aptamer with sbC-PEGylation. **1**: 2-cyanoethyl-*N,N*-diisopropyl (CED) phosphoroamidite; **3**: DMT-triethyloxy-glycol phosphoramidite; **5**: ssH linker phosphoramidite; **7**: dimethyl sulfoxide-acetonitrile solution of activated PEG; DMTr: 4,4´-dimethoxytrityl; MMTr: *p*-methoxyphenyl diphenylmethyl; OCE: cyanoethoxy.

As an alternative conjugated polymer, monoselenide-containing polymer PEG-PUSe-PEG was synthesized with high sensitivity to oxidation stimuli.[51] PEG-PUSe-PEG is an amphiphilic triblock copolymer with hydrophobic monoselenide polyurethane blocks and hydrophilic poly(ethylene glycol) (PEG) blocks (Figure 11).

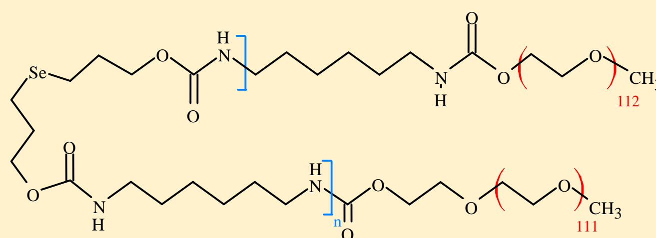

Figure 11. The structure of monoselenide-containing polymer PEG-PUSe-PEG.

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6.3.4.1. The proposed synthetic procedure of the multifunctional hybrid nanoparticles

The proposed synthetic procedure of multifunctional hybrid nanoparticle is shown in Figure 12. Briefly, the synthesis of nanoflowers can be accomplished by decomposition of manganese acetylacetonate [Mn(acac)$_2$] in diphenyl ether in the presence of preformed gold nanoparticles (Au NPs) ("seeds"), with oleic acid and oleylamine as surfactants.[52,53] The gold "seeds" can be generated in situ by decomposition of gold acetate [Au(OAc)$_3$] at low temperature, which could be traced by a color change to deep red. The formation of MnO petals can be achieved by epitaxial growth on the surface of the Au NPs at higher temperatures. The size and morphology of the nanoflowers can be varied by changing the molar ratio of the precursors, while the number and size of the MnO petals can be increase with increasing [Mn(acac)$_2$]/[Au(OAc)$_3$] ratio.[53]

These Au@MnO nanoflowers should be water-soluble, and this can be achieved either by using dopamine-PEG-COOH biopolymer or by introducing a quaternary ammonium. Synthesis of this heterobifunctional ligand, dopamine-PEG-COOH, can be carried out with the three following steps: (i) synthesis of 3,4-dihydroxyhydrocinnamic acid pentafluorophenol ester, (ii) synthesis of NH$_2$-PEG-COOH, and (iii) synthesis of dopamine-PEG-COOH.[54]
Whereas introducing a quaternary ammonium can be accomplished via two pathways. The addition of the ligand 3-sulfanylpropyltrimethoxysilane **1** to the nanoflowers in propanol, in which the thiol would bind to the particle surface and the trimethylsilyl group would be available for further functionalization. Then, polycondensation in the presence of relatively small amount of 3-aminopropyl-dimethylethoxysilane ligand **2** and ligand **3** functionalized with quaternary ammonium in a ratio of 1:1 would yield multi-functionalized Au@MnO nanoflowers. The amino group of ligand **2** can be readily employed for coupling reactions with biological molecules (Figure 13). Whereas quaternary ammonium groups of ligand **3** cannot bind to aptamers, they only provide a positive charge to the particles over the entire pH range and give rise to the desired water solubility of the particles during the coupling with aptamers.[55]

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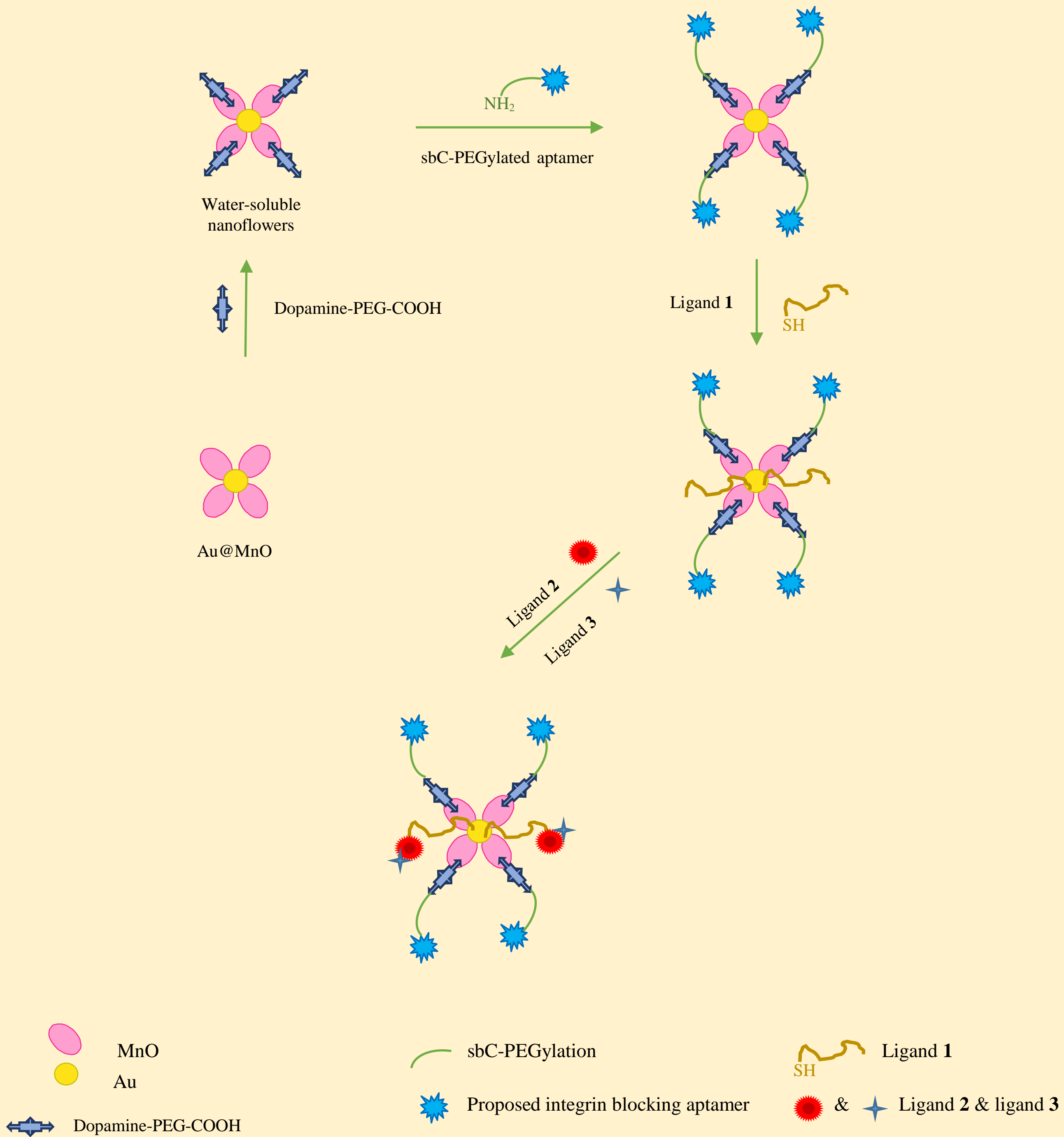

Figure 12. Schematic illustration of surface functionalized of Au@MnO nanoflowers with both aptamers and different ligands.

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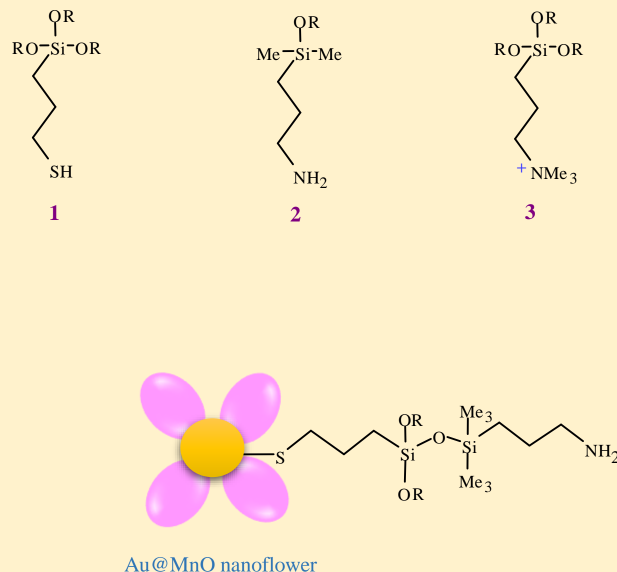
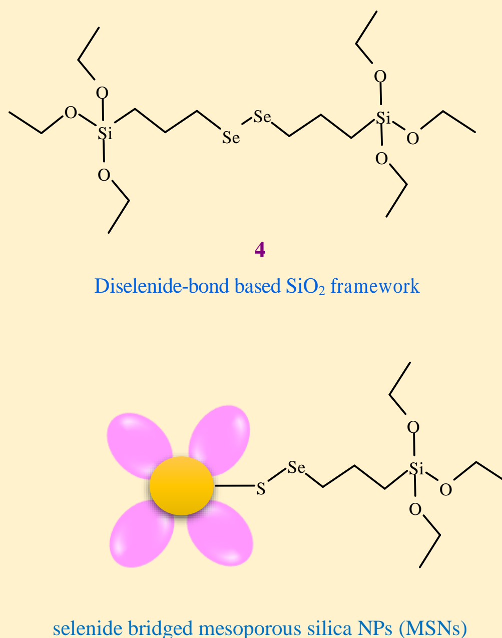


Figure 13. Schematic composition of the ligand shell.

Moreover, diselenide-bond-containing organosilica moieties, e.g. compound **4**, can be incorporated into the silica network to fabricate a dual responsive selenide bridged mesoporous silica NPs (MSNs). The complex compounds such as *cis*-β-hydroxoaquatriethylenetetramine cobalt(III), and 2-hydroxmethyl phenanthroline-Zn^{2+} can then encapsulate into the internal pores of MSNs through electrostatic interactions. Upon exposure to oxidative or reductive conditions, the MSN frameworks degraded owing to the cleavage of diselenide bonds, and the complex compound can consequently be released.



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6.3.4.2. The expected results of this hypothetical approach

The expected results of aptamer-functionalized Au@MnO nanoflowers can be explained as follow. First, the aptamers have the ability to inhibit integrin-ligand binding, and integrin-mediated TGF-β signaling. Furthermore, they are able to recognize the active conformational epitopes, and may influence the conformational environment of integrin molecules. Second, the incorporated complex compounds can effectively hydrolyze the phosphorylated SMADs, and ATP. Third, the incorporated metal cations may disturb the local hydrogen bonding interactions and conformational dynamics of the hinge region of protein kinase leading to weakening the long-range interdomain correlations to favor the open conformation. Finally, the participation of selenium-containing polymer has the ability to dissociate the disulfide linked homodimer of LAP leading to blocking TGF-β signaling.

7. Conclusions

This present study describes new proposed strategies for selective neutralization of TGF-β signaling. The study highlights how the mode of action of TGF-β signaling is; the relative activities of protein kinase and protein phosphatases; and the influence of the myristoylation and the cellular senescence-inhibited gene on protein phosphatase. Moreover, this study describes the convergence between TGF-β and other pathways manifesting the multifaced activities of TGF-β, as well as its implication in various diseases. Additionally, this study describes the conformation of protein kinases, and how the uncontrollable spatial and temporal phosphorylation of these proteins can cause devastating consequences.

In the light of these aspects, I proposed new strategies for the selective neutralization of TGF-β signaling. These strategies are based on that: 1) the coordination of divalent metal cations (M^{2+}) may imply the stability of the active conformation of the kinase. Metal cations include Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, that have the ability to implement such a role alone or in the presence of an organic molecule. 2-hydroxymethyl phenanthroline-M^{2+} may stimulate ATP hydrolysis, and may also inhibit the phosphorylation process of SMADs; 2) the metal complex compound "*cis*-β-hydroxoaquatriethylenetetramine cobalt(III)" may hydrolyze the RGD motif of the LAP chain blocking the affinity of αv integrin to the RGD sequence; 3) the dissociation of the disulfide linked homodimer of the LAP peptides by the catalytic activity of selenols may represent a potent option for neutralizing TGF-β signaling; 4) an aptamer-functionalized Au@MnO nanoflowers can serve as a multifaced therapeutic agent targeting TGF-β signaling. This novel proposed agent allows to contain different synergistic molecules, which can perform combined activities leading to produce a joint effect to regulate the pleiotropic cytokine TGF-β signaling; and 5) An integrin blocking aptamer may play a substantially role in the inhibition of integrin-mediated latent TGF-β activation as well as its downstream signaling.

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