Protein-guided Self-Assembly for Controlling Protein–Protein Interactions

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Abstract: Synthetic agents that disrupt intracellular protein–protein interactions (PPIs) are desirable for elucidating cellular signaling networks and for development of new therapeutics. However, designing large cell-permeable molecules that are equipped with many functional groups necessary for binding to protein interfaces still remains a challenge. Recent studies of self-assembling small molecules have shed light on the robustness of approaches based on artificial synthetic platforms towards protein surface recognition with a wide range of applications, including library synthesis, protein sensing, and in situ generation of bioactive molecules. This review illustrates recently developed strategies of protein-templated assembly, in which self-assembly of small molecules is induced by binding to a targeted protein. Following chemical reactions between the fragments results in generation of a conjugated molecule, which is capable of modulating functions of water-exposed protein surfaces.

Keywords: inhibitors, kinetic target-guided synthesis, protein template, protein–protein interactions

Introduction

Regulation of intracellular protein–protein interactions (PPIs) by synthetic agents has become a major challenge in the post-genome era as dysregulated proteins found in diseases are often implicated in intracellular signaling circuits[1]. However, structurally diverse interfaces of PPIs limit development of cell-permeable agents, because various functional groups need to be precisely allocated for specific binding to the surfaces through multivalent interactions, therefore require large molecular size[2]. Molecules ranging in molecular weight between roughly 600 and 2,000 have been disregarded by pharmaceutical industries because many of them do not conform to Lipinski’s rule of five for drug-likeliness. Nonetheless, their large molecular surface would allow for the introduction of multivalency and structural diversity, characteristics that are necessary for protein surface recognition. In addition, such mid-sized agents are attractive as they may allow us to decrease the molecular weight by more than two orders of magnitude compared to protein-based biologics, and thus increase cell penetration and plasma stability. Synthetic tractability would also reduce the production cost. We[3] and others[4] have developed mid-sized molecule-based synthetic agents that are capable of regulating PPIs, however, several issues must be addressed for development of the large-sized inhibitors to be successful. One question is how to reduce the molecular size to ensure cell permeability while retaining binding affinity and selectivity. We envisioned that one potential solution might be utilizing kinetic target-guided synthesis (KTGS)[5]. In this approach, a mid-sized agent is generated by covalent linking of
two small molecules by exploiting the template effect of a target protein surface. For example, two reactive small-module compounds are designed to bind side-by-side to a targeted protein surface, and upon binding, the resulting proximity effect would trigger a chemical ligation, producing a covalently conjugated compound that is predicted to be a better PPI inhibitor than either module compound alone (Figure 1). This review illustrates earlier examples of KTGS that facilitate identification of synthetic protein ligands, followed by our recent effort on an application of KTGS to intracellularly generate a mid-sized agent which significantly suppress cell growth.

**Templated ligation**

The pioneering study of the template ligation reaction was reported in 1969[6]. In this study, carnitine acetyltransferase, an enzyme which transfers an acetyl group from acetyl-CoA to carnitine to produce O-acetylcarnitine, was used, demonstrating that the enzyme’s activity is inhibited in the presence of bromoacetyl derivatives of CoA and carnitine, presumably due to the covalent linking of CoA and the carnitine moiety, and the resulting conjugate binds tightly to the enzyme’s active pocket. In 1986, Readout reported self-assembly to generate a toxin, by a condensation reaction of decanal and N-amino-N'-1-octylguanidine (AOG) producing the corresponding detergent-like hydrazine, a potent cytotoxicin[7]. A mixture of decanal and AOG showed significant cytotoxicity, whereas either decanal or AOG alone was inactive, confirming the in situ assembly of the more toxic product.

**KTGS by click chemistry**

In 2002, Sharpless and Finn and co-workers introduced the copper-catalyzed azide-alkyne cycloaddition (“click chemistry”) reaction for KTGS[8]. The first example of target-templated reaction was a click chemistry with acetylcholinesterase (AChE)[9]. The site-specific ligands tacrine and phenanthridinium were attached to either alkyl azide or alkyl acetylene group via various spacers to give two respective sets of building blocks. These compounds were incubated in the presence of AChE, and the mixtures were then analyzed. Although the reactions were expected to produce nearly 100 potential bivalent compounds, only one combination gave an adduct. Importantly, the yield of product in the absence of enzyme was negligible. The product syn-1 was found to be the most potent noncovalent AChE inhibitor (Figure 2). The isomer anti-1 was also a strong ligand but exhibited lower binding affinity than syn-1, indicating that the triazole ring location within the enzyme pocket is critical for binding.
cause of the low rate of background reaction, the resulting adduct can be used as a lead compound for development of inhibitors of the target enzyme used as the template for the in situ click chemistry reaction. This study was further expanded to test a larger library of acetylene building blocks linked to various heterocycles, leading to the identification of derivative (2), which exhibited a remarkable femtomolar dissociation constant [9].

KTGS based on in situ click chemistry has been utilized to identify inhibitors of a number of enzymes. Examples include carbonic anhydrase [10], HIV protease [11], chitinase [12], acetylcholine-binding proteins [13], tyrosine kinase Abl [14], transcriptional repressor EthR [15], myelin-associated glycoprotein [16], and histone deacetylase [17].

In 2009, Heath and co-workers applied TGS to large protein surfaces, using building blocks exhibiting moderate affinity [18]. The strategy was designed for identifying large peptide-based agents that bind to the surface of bovine CA II (bCA II). First, a one-bead-one-compound library of heptapeptides comprised of D-amino acids and an acetylene-containing amino acid (D-propargylglycine, D-Pra) was screened for binding to bCA II. The identified hit was LKLWFK-(D-Pra), which exhibited only weak affinity (0.5 mM) for bCA II. The click reaction was then initiated to produce a 14-mer peptide derivative exhibiting affinity that was higher than that of the first heptapeptide fragment by two orders of magnitude ($K_d \sim 3 \mu M$). This bivalent compound was subjected to a third round of ligand screening, which resulted in the identification of a tridentate ligand containing 21 amino acid residues that exhibited much-improved affinity (approximately 50 nM).

**KTGS for PPI-directed library synthesis**

In situ assembly to generate multivalent large molecules that are capable of binding to the interfaces may provide a useful platform for the development of PPI inhibitors. In 2008, Manetsch and co-workers first applied the KTGS strategy for PPI-directed library synthesis using Bcl-XL protein as a template [19]. The Bcl-2 family protein Bcl-XL is often overexpressed in cancers and is responsible for drug resistance. Inhibiting Bcl-XL to enhance apoptotic responses is regarded as a promising strategy for anticancer drugs. In this study, a shallow binding groove of Bcl-XL was exploited for the target-guided in situ assembly of two fragments, which was driven by the sulfonamide formation reaction between thio acids and azides proceeding under mild conditions in aqueous solution (Figure 3) [20, 21]. Each combination of sulfonyl azides and thio acids was treated with Bcl-XL, and found that only one gave an increased amount of acylsulfonamide (SZ4TA2) in the presence of the protein. A fluorescent polarization competition assay using Bcl-XL and a fluorescein-labeled Bak BH3 peptide revealed that SZ4TA2 inhibits the binding of the peptide to Bcl-XL at low nanomolar concentrations. These results confirm that in addition to enzyme active sites, kinetic TGS can also be used with shallow binding grooves at PPI interfaces.

**Synthesis of inhibitors of protein-protein interactions in cells**

Synthetic inhibitors for PPIs are often large in size and therefore tend to suffer from cell penetration. We hypothesized that a strategy based on intra-
Figure 4. Left: Chemical structure of the naturally occurring 14-3-3 ligands fusicoocin and the phosphopeptide, PMA2. Right: Crystal structure of the ternary complex of 14-3-3, FC, and PMA2.

Figure 5. (A) Generation of 14-3-3 inhibitor 5 by oxime ligation between the reactive modules (3 and 4). (B, C) Close look (B) and overview (C) of a docking model of 3 and 4 in 14-3-3ζ. (D) A docking model of 5 and 14-3-3ζ.
cellular self-assembly would provide a solution to this problem if two reactive motifs designed for TGS are capable of penetrating cells. We performed a proof-of-concept study focusing on 14-3-3 proteins as primary targets[22]. The 14-3-3 proteins are dimeric regulatory proteins that play crucial roles in the regulation of serine/threonine kinase-dependent signaling pathways. Each monomer possesses a shallow binding groove that recognizes phosphoserine or phosphothreonine residue-containing consensus motifs that are found in at least 200 different 14-3-3 partner proteins. Dysregulation of 14-3-3 interactions have been linked to many diseases, including cancers and neurological diseases, suggesting that 14-3-3 is a potential therapeutic target[23].

Diterpene phytotoxic fusicoccin (FC) is known to enhance 14-3-3 binding to the PMA2 peptide (QSYpTV), which is the C-terminal 14-3-3 motif of plant H⁺-ATPase, by forming a ternary complex (Figure 4). A study based on the crystal structure of the ternary complex demonstrated that FC and the phosphopeptide bind cooperatively through van der Waals interactions between FC and the valine residue of the peptide, leading to complete occupation of the long, shallow binding groove[24]. The crystal structure, along with the fact that 14-3-3 proteins are relatively abundant in cells, prompted us to test a TGS approach to covalently link the natural product to the peptide motif and generate a large conjugate as an intracellular 14-3-3 inhibitor.

Structure-based design of the two reactive motifs for the TGS based on the crystal structure of the ternary complex led to compounds 3 and 4, in which an aldehyde and an oxyamino group, respectively, were introduced for oxime ligation (Figure 5A). A computational simulation showed that the benzyl group at the 12-position of FC favorably projects the aldehyde at the ortho position toward the ammo-

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Figure 6. HPLC profiles of the reactions in the absence (left) and presence (right) of 14-3-3.

Figure 7. Left: Anti-proliferative activity of chemically synthesized 5 and a mixture of 3 and 4. Right: Western blot of coimmunoprecipitated proteins using anti-cRaf and -FLAG antibodies.
nium group of 4, which forms a hydrogen bond with the aldehyde oxygen, suggesting that the aldehyde and ammonium groups readily react to give the desired oxime derivative (Figure 5B). The modeling also suggested that the resulting conjugate (5) has an appropriate shape for binding to 14-3-3 (Figure 5C and D).

In vitro evaluation of the ligation by HPLC demonstrated that the ligation was significantly accelerated by 14-3-3 (Figure 6), and the resulting compound (5) possesses sub-micromolar binding affinity for 14-3-3[22]. Furthermore, cell-based experiments revealed that the ligation proceeded in cells, resulting in significant cytotoxicity (Figure 7, left). No apparent cytotoxicity was observed for 3 or 4, supporting that these motifs are only weak ligands for 14-3-3. Importantly, chemically synthesized 5 was found to be even less toxic than 3 and 4, suggesting that the large conjugate 5 lacks the ability to penetrate cells. The cytotoxicity observed for the equimolar mixture of 3 and 4 was abolished when 3 was substituted with an analogue lacking the aldehyde, confirming that intracellular generation of 5 is requisite for the activity[22]. Finally, an immunoprecipitation experiment demonstrated that 5, but not 3 or 4, disrupts binding of 14-3-3 and c-Raf protein, confirming that the intracellularly generated ligation product is capable of inhibiting 14-3-3 PPIs, thus resulting in cytotoxicity. This proof-of-concept study suggests that intracellular self-assembly followed by in situ TGS may provide insights into potential pro-drug strategies for controlling intracellular signaling pathways.

Summary

Methodologies based on protein-guided self-assembly have developed and provided a versatile platform for generation of protein surface-directed combinatorial libraries, tools for protein sensing, and PPI inhibitors. Although examples are still limited, intracellular generation of self-assembled agents is of interest for further application. Future studies may also expand to control dynamic events, such as signaling processes that are regulated by disordered proteins.

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