Peptide drug leads with increased inhibitory properties synthesized using a divergent strategy

Review Article

Protein-protein interactions (PPI) play a major role in regulating many cellular processes, which makes them attractive druggable targets. The large surface area involved in PPIs, however, demands high selectivity of large drug leads such as proteins that can be difficult to modify and fine-tune. To demonstrate a solution to this problem, Christian Tornøe and his colleagues at Novo Nordisk in Denmark have synthesized analogues of Bowman-Birk protease inhibitor (BBI) by using native chemical ligation of peptide hydrazides to link together peptide building blocks to generate several analogues of BBI. This approach required fewer reaction steps than a linear synthesis strategy, and could be used to graft a specific region of a potent trypsin inhibitor onto the α-chymotrypsin-binding loop of BBI that boosted its inhibitory effect four-fold.

A strategy to efficiently generate BBI analogues aimed at boosting protease inhibition

Bowman-Birk Inhibitor protein (BBI) is a protease inhibitor of 71 amino acids, of which 20% are cysteines, that protects seeds against insects and pathogens. While not covered in this publication, studies in animal models have also shown that dietary BBI from several legume sources can prevent or suppress carcinogenic and inflammatory processes within the gastrointestinal tract, which has stimulated the investigation of BBI as a potential colorectal chemopreventive agent (see, for example, Clemente, A, and del Carmen Arques, M, 2014).

BBI has two hairpin loops, each consisting of a disulfide-linked nine-residue loop, which project from the core and inhibit trypsin and α-chymotrypsin. The research team at Novo Nordisk tried to increase the inhibitory activity of BBI by using it as a scaffold to synthesize analogues through native chemical ligation and then fold the cysteine-rich proteins to form a series of BBI analogues ready for *in vitro* testing. Their divergent synthesis strategy enabled them to synthesize four analogues using a total of six ligation steps that involved five peptide segments (Figure 1). By contrast, a linear strategy without intermediates would require eight ligation steps.

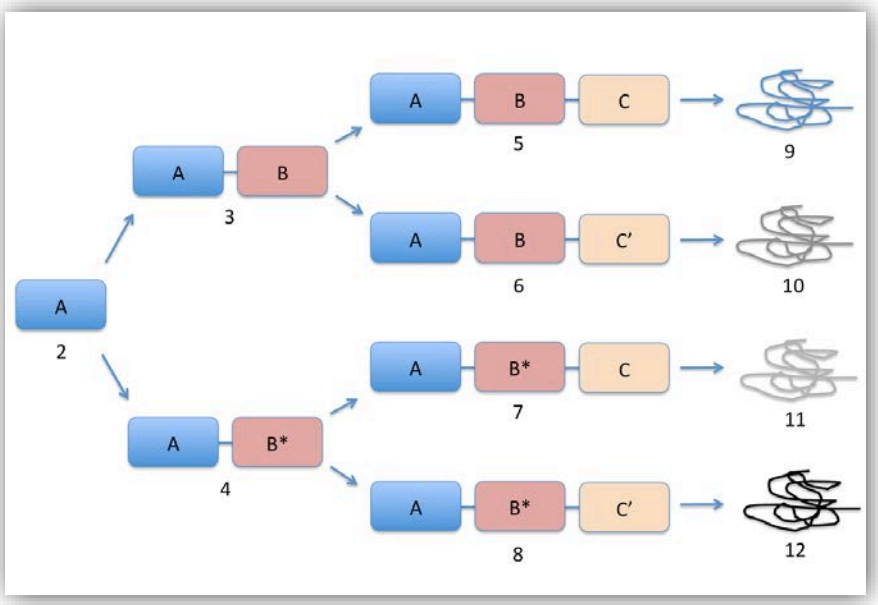

Figure 1. *The analogues were prepared using five peptides, A, B, B*, C, and C', where * means a mutation and ' means a truncation. These peptides were linked in the configurations shown to give proteins* ***5****,* ***6****,* ***7****, and* ***8****, which were then folded to give proteins* ***9****,* ***10****,* ***11****, and* ***12****, respectively.*

Synthesis chemistry

BBI peptide segments were synthesized by solid-phase peptide synthesis with DIC/Oxyma Pure® activation on a Prelude® peptide synthesizer, followed by cleavage and removal of protecting groups with trifluoroacetic acid. The peptides were purified using reverse-phase HPLC, with yields in the 8–40% range.

The peptides were used to construct the full-length proteins (Figure 1) through the latent thioester properties of the C-terminal hydrazides. For example, the N-terminal of peptide segment A (**2**) was activated with sodium nitrite and sodium 2-mercaptoethanesulfonate to create a thioester that could be reacted with B and B* to give **3** and **4,** respectively. These were divided and reacted with C and C' to produce **5**, **6**, **7**, and **8**. Mutations were included in the middle and C-terminal segments of BBI. Met27 on the surface of the protein was replaced with Leu27 to avoid oxidation, and no mutations were placed in the trypsin-binding loop. Once synthesized, the proteins were folded at high dilution and only required buffer exchange before being tested *in vitro*.

The researchers also synthesized a small potent trypsin inhibitor, SFTI-1 (**13**), and a variant, [Phe5]-SFTI-1 (**14**) for comparison purposes.

Inhibition increased by grafting selected residues of a known trypsin inhibitor

A number of differences between the analogues were noted when testing their inhibitory properties, but the most significant improvement was seen in the BBI analogue (**11**). Its design was based on the observation that the α-chymotrypsin binding loop of BBI appears to be suboptimal whereas SFTI-1 has higher binding. This stimulated the researchers to graft four residues from [Phe5]-SFTI-1, a potent α-chymotrypsin inhibitor, onto the α-chymotrypsin binding loop to create a BBI/SFTI-1 chimera (**11**). The result was a four-fold increase in inhibition over native BBI, from 59 nM to 16 nM.

Folding and binding confirmed by X-ray crystallography

X-ray crystallography showed that the BBI analogue (**11**) had the same folding and binding to α-chymotrypsin as native BBI, but Phe43 bound deeper in the S1 pocket of α-chymotrypsin as compared to the Leu43 of BBI, which might explain the increased inhibition. The researchers also used dynamic light scattering to discover that truncating the C-terminal, for example in analogue (**10**), increased self-association.

A new approach to the high throughput generation of protein diversity

Druggable targets such as those involved in protein-protein interactions require the high selectivity provided by large molecules such as proteins. While high throughput modification and screening of small molecules is well established, the high-throughput generation of protein diversity is more demanding, and requires flexible expression systems and protein purification, or efficient chemical synthesis. The strategy of divergent protein synthesis reported here is clearly an attractive alternative for efficient generation of the molecular diversity needed to explore protein-protein interactions and other druggable targets.

References

Divergent Protein Synthesis of Bowman-Birk Protease Inhibitors, their Hydrodynamic Behavior and Co-crystallization with α-Chymotrypsin. Tornøe C, et al. Synlett 2017; 28(15): 1901-1906. DOI: 10.1055/s-0036-1588840.
https://www.thieme-connect.com/products/ejournals/html/10.1055/s-0036-1588840

Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents. Clemente, A, and del Carmen Arques, M. World J Gastroenterol. 2014 Aug 14; 20(30): 10305–10315. Published online 2014 Aug 14. doi: 10.3748/wjg.v20.i30.10305.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4130838/