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Research paper

Engineering potent mesotrypsin inhibitors based on the plant-derived cyclic peptide, sunflower trypsin inhibitor-1

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ABSTRACT

Plants produce a diverse range of peptides and proteins that inhibit the activity of different serine proteases. The value of these inhibitors not only stems from their native role(s) *in planta*, but they are also regarded as promising templates for inhibitor engineering. Interest in this field has grown rapidly in recent years, particularly for therapeutic applications. The serine protease mesotrypsin has been implicated in several cancers, but is a challenging target for inhibitor engineering as a number of serine protease inhibitors that typically display broad-range activity show limited activity against mesotrypsin. In this study, we use a cyclic peptide isolated from sunflower seeds, sunflower trypsin inhibitor-1 (SFTI-1), as a scaffold for engineering potent mesotrypsin inhibitors. SFTI-1 comprises 14-amino acids and is a potent inhibitor of human cationic trypsin ($K_i = 30 \pm 0.8$ pM) but shows 165,000-fold weaker activity against mesotrypsin ($K_i = 4.96 \pm 0.2$ μM). Using an inhibitor library based on SFTI-1, we show that the inhibitor's P2′ residue (Ile) is a key contributor to SFTI-1's limited activity against mesotrypsin. Substituting P2′ Ile with chemically diverse amino acids, including non-canonical aromatic residues, produced new inhibitor variants that maintained a similar structure to SFTI-1 and showed marked improvements in activity (exceeding 100-fold). An assessment of the activity of the new inhibitors against closely-related trypsin paralogs revealed that the improved activity against mesotrypsin was accompanied by a loss in activity against off-target proteases, such that several engineered variants showed comparable activity against mesotrypsin and human cationic trypsin. Together, these findings identify potent mesotrypsin inhibitors that are suitable for further optimisation studies and demonstrate the potential gains in activity and selectivity that can be achieved by optimising the P2′ residue, particularly for engineered SFTI-based inhibitors.

1. Introduction

In humans, three trypsin paralogs are produced by the pancreas and secreted into the duodenum where they assist with protein digestion. The two most abundant paralogs, cationic trypsin and anionic trypsin, are broadly distributed among vertebrates and have served as model enzymes for investigating the structure and function of serine proteases. However, the third paralog, mesotrypsin, stands apart due to its relatively recent evolution and unusual biochemical traits. Mesotrypsin is found in relatively few species; the gene encoding mesotrypsin (*PRSS3*) emerged in hominids after they diverged from Old World monkeys [1]. This gene was formed by duplicative transfer of genetic material from two separate chromosomes and gives rise to several distinct transcripts as the initiation sequences from both parental gene segments have been retained [1]. The first two mesotrypsin transcripts to be reported were identified in cDNA libraries derived from the pancreas and brain, with each transcript yielding an active protease that has identical amino acid sequence.

Mesotrypsin also has several unusual traits that relate to its enzymatic activity. The most remarkable is that mesotrypsin is resistant to a number of proteinaceous trypsin inhibitors, even though it shares almost 90% amino acid sequence identity to cationic trypsin or anionic trypsin [2,3]. For example, aprotinin and soybean trypsin inhibitor are well-known inhibitors of cationic and anionic trypsin, but these inhibitors show weak activity against mesotrypsin and, instead, appear to be substrates for this enzyme [2,4]. Mesotrypsin's atypical enzymatic activity suggests that it

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; KLK, kallikrein-related peptidase; MCA, 4-methylcoumaryl-7-amide; SFTI-1, sunflower trypsin inhibitor-1.

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might influence proteolytic networks in a different way to other serine proteases. This attribute has potential relevance to a variety of physiological functions as recent studies have shown that mesotrypsin is expressed by a wider range of tissues and cell types than first thought, including a new splice variant that is expressed by differentiating keratinocytes [5]. One cell type of particular interest is cancer cells as overexpression of mesotrypsin has been identified in several cancers, including pancreatic cancer [6], prostate cancer [7] and ovarian cancer [8]. Although its precise role and key substrates remain to be fully identified, elevated expression of mesotrypsin in patient tumours has been associated with poor prognosis [6–8] and mesotrypsin has been shown to promote tumour growth and metastasis in various cancer models [6,7].

Mesotrypsin's resistance to protein-based inhibitors that are effective against other trypsin paralogs has been studied from both structural and biochemical perspectives. Mesotrypsin adopts a classic chymotrypsin fold (clan PA) and is identical to human cationic trypsin in 196 out of 224 residues (Fig. 1A–B) [9–11]. However, a small number of point mutations exist within the active site cleft of mesotrypsin that convey resistance to trypsin inhibitors. The most striking mutation is Gly193Arg, which alters a Gly residue that is conserved in almost all other proteases in the S1A family [9]. The importance of this mutation was established by studying the activity of a mesotrypsin mutant where Arg193 was substituted back to Gly, which restored the enzyme's sensitivity to trypsin inhibitors [2]. Although Arg193 mainly conveys mesotrypsin's resistance to trypsin inhibitors, a recent study that examined critical mutations for converting human cationic trypsin into a mesotrypsin-like enzyme has shown that additional residues contribute to the enzyme's unusual activity [3]. In that study, three additional mutations, Tyr39Ser, Glu74Lys and Lys97Asp, together with Gly193Arg (Fig. 1A–B), were found to be necessary to fully transfer mesotrypsin's unique biochemical traits to human cationic trypsin. Other studies have shown that the level of activity that a given inhibitor displays against mesotrypsin also varies depending on the inhibitor's broader sequence and structure [4,12]. As a result, the efficiency by which mesotrypsin cleaves inhibitors is not only determined by the sequence of the binding loop, but residues within the inhibitor's scaffolding also influence its activity [4,12]. Together, insights from these studies have helped to guide the engineering of several protein-based mesotrypsin inhibitors that show higher activity and are less susceptible to cleavage by mesotrypsin [13,14].

Plant-derived serine protease inhibitors are a diverse group of proteins that have excellent credentials as templates for designing inhibitors for new protease targets. For engineering applications, the variety in size and structure evident among plant-derived protease inhibitors is beneficial as it provides an ensemble of optimised scaffolds that are suitable for different targets. An unusual feature found in several inhibitors is a cyclic peptide backbone whereby the N- and C-termini are connected via an amide bond. One group of inhibitors that possess this trait are the PawS-derived peptides that are produced during preproalbumin maturation in *Helianthinae* plants [15,16]. The first member of this family to be characterised was sunflower trypsin inhibitor-1 (SFTI-1), a 14-amino acid cyclic peptide that is stabilised by a single disulfide

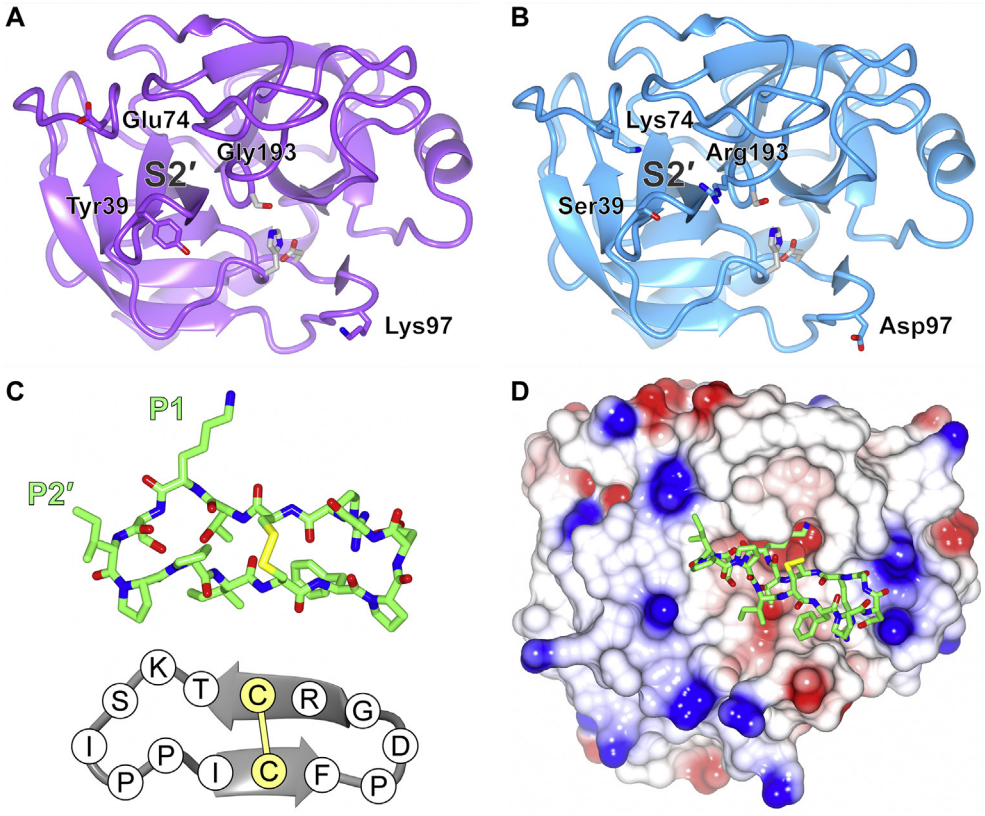

Fig. 1. Structures of trypsin, mesotrypsin and sunflower trypsin inhibitor-1 (SFTI-1). (A) Structure of human cationic trypsin (purple, PDB ID 2RA3) and (B) structure of mesotrypsin (blue, PDB ID 1H4W). Both proteases are shown as ribbon diagrams (in the same orientation) and the serine protease catalytic triad (His, Asp, Ser) is shown in stick model (carbon atoms: silver). Point mutations identified to contribute to mesotrypsin's unique activity (Ser39, Lys74, Asp97 and Arg193) are illustrated in panel B, with the corresponding residues in human cationic trypsin shown in panel A. (C) Structure of SFTI-1 (PDB ID 1SFI) shown in stick model with the P1 and P2′ residues labelled. The amino acid sequence of SFTI-1 (single letter code) is overlayed on a ribbon diagram of SFTI-1. Backbone cyclisation occurs between Gly1 and Asp14 *in planta*. (D) Model of SFTI-1 bound to mesotrypsin (see section 5.6 for further details). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)