Mastering the Canonical Loop of Serine Protease Inhibitors: Enhancing Potency by Optimising the Internal Hydrogen Bond Network

Joakim E. Swedberg1, Simon J. de Veer1, Kei C. Sit1, Cyril F. Reboul2, Ashley M. Buckle2, Jonathan M. Harris1*

1 Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia 2 Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Faculty of Medicine and Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia

Abstract

Background: Canonical serine protease inhibitors commonly bind to their targets through a rigid loop stabilised by an internal hydrogen bond network and disulfide bond(s). The smallest of these is sunflower trypsin inhibitor (SFTI-1), a potent and broad-range protease inhibitor. Recently, we re-engineered the contact β-sheet of SFTI-1 to produce a selective inhibitor of kallikrein-related peptidase 4 (KLK4), a protease associated with prostate cancer progression. However, modifications in the binding loop to achieve specificity may compromise structural rigidity and prevent re-engineered inhibitors from reaching optimal binding affinity.

Methodology/Principal Findings: In this study, the effect of amino acid substitutions on the internal hydrogen bonding network of SFTI were investigated using an in silico screen of inhibitor variants in complex with KLK4 or trypsin. Substitutions favouring internal hydrogen bond formation directly correlated with increased potency of inhibition in vitro. This produced a second generation inhibitor (SFTI-FCQR Asn14) which displayed both a 125-fold increased capacity to inhibit KLK4 ($K_i = 0.0386\pm 0.0060$ nM) and enhanced selectivity over off-target serine proteases. Further, SFTI-FCQR Asn14 was stable in cell culture and bioavailable in mice when administered by intraperitoneal perfusion.

Conclusion/Significance: These findings highlight the importance of conserving structural rigidity of the binding loop in addition to optimising protease/inhibitor contacts when re-engineering canonical serine protease inhibitors.

Introduction

Prostate cancer is the most commonly diagnosed male cancer in western countries, accounting for more than 32,000 deaths last year in the United States alone [1]. Although current treatments for localized prostate cancer are highly successful, less than one third of patients with metastatic disease survive five years following diagnosis [1]. This emphasises the urgent need for effective treatments for patients suffering from late stage disease. Prostate cancer is primarily detected using serum levels of kallikrein-related peptidase 3 (KLK3, prostate-specific antigen, PSA), which is the established biomarker for diagnosis and prognosis [2]. KLK3 belongs to the kallikrein-related peptidase (KLK) multi-gene family which encodes fifteen homologous serine endopeptidases with trypsin or chymotrypsin-like substrate specificity. It is well documented that KLK proteases significantly contribute to several important (patho)physiological functions [3]. Consequently, there is a growing interest in the utility of KLKs in certain pathologies as biomarkers and therapeutic targets [4,5], particularly in hormone-dependent cancers [6].

One KLK of interest, KLK4, is principally expressed in basal and secretory cells of the prostate gland and is commonly overexpressed in malignant prostate tumours [7,8]. Recent studies indicate that the proteolytic activities of KLK4 closely align with events central to cancer development and progression. Firstly, KLK4 has been shown to degrade components of the extracellular matrix in vitro [9], as well as cleave insulin-like growth factor binding protein 3-6 [10] and urokinase plasminogen activator receptor [11]. Secondly, cell culture experiments have demonstrated that KLK4 enhances a diverse array of strongly tumourigenic functions. Of note, KLK4 stimulates protease-activated receptors −1 and −2 which are also overexpressed in prostate cancer, resulting in cytoskeletal remodelling and increased cell migration and proliferation [12,13,14]. These findings complement earlier studies which found overexpression of KLK4 was associated with an epithelial-to-mesenchymal transi-
tion in prostate cancer cells [8] and that KLK4 may modulate interactions between tumour cells and osteoblasts in the development of bone metastases [15]. Therefore, targeted inhibition of KLK4 may present an avenue to new treatments for advanced prostate cancer.

It has previously been reported that the naturally occurring sunflower trypsin inhibitor (SFTI-1) inhibits KLK4 [16], in addition to known targets such as trypsin [17], cathepsin G [18] and matrilysin (ST14/MT-SP1) [19]. SFTI-1 is a 1.4 kDa cyclic Bowman-Birk serine protease inhibitor (BBI) isolated from sunflower (Helianthus annus) seeds. Its three-dimensional structure in complex with trypsin [17] reveals a dual anti-parallel β-sheet arrangement stabilized by a disulfide bridge and an extensive internal hydrogen bonding network [20]. SFTI-1 binds to target proteases by an extended β-sheet across the P1–P4 residues to form a tight binding complex (trypsin/SFTI-1 \( K_i = 0.1 \) nM) [21]. This mode of binding is not only common to canonical serine protease inhibitors [22] but forms the basis of protein substrate and inhibitor recognition across all families of proteases [23].

Another important feature of SFTI-1 is that its scissile bond (P1–P1') can be cleaved and reformed with an equilibrium of 1:9 in favour of the intact bond [24]. This phenomenon is evident in at least 19 convergently evolved serine protease inhibitor families [25] and is referred to as ‘standard’ or Laskowski mechanism of inhibition [26,27]. Standard mechanism binding loops are typified by a high degree of rigidity due to an internal network of stabilising hydrogen and disulfide bond(s). This has particular significance to inhibitor function; not only does it allow for a lower entropic debt upon protease binding [28], it also permits formation of an acyl-enzyme intermediate with largely unchanged conformation [29,30]. As the products of hydrolysis are still associated with the protease, resynthesis of the peptide bond is more favoured than the intermolecular reaction with lower local reactant concentrations [30,31].

To harness the favourable structural features of SFTI-1 and redirect inhibition towards KLK4, the contact β-sheet of SFTI was recently re-engineered using a sparse matrix peptide library to guide amino acid substitutions. The resulting inhibitor, SFTI-FCQR (P1 Lys to Arg, P2 Thr to Gln and P4 Arg to Phe) selectively inhibited KLK4 (\( K_i = 3.59 \pm 0.28 \) nM) and uniformly showed low inhibition of other SFTI-1 targets and closely related KLKs [16]. However, the constrained geometry of SFTI prevents the use of linear peptide libraries to optimise interactions beyond KLKs [16]. The most favourable substitution produced a second generation inhibitor with a binding affinity for KLK4 similar to that of SFTI-1 for trypsin while selectivity was markedly improved. These findings underline the importance of binding loop rigidity in canonical serine protease inhibitors and the need to maintain structural stability when modifying compounds of this class.

**Methods**

**Protein expression and purification**

Recombinant KLK4 and KLK14 were produced using Sf9 insect cell expression constructs as previously reported [18,16]. These expression vectors generate the complete KLK amino acid sequence followed by a V5 epitope (GKPIPNPLLGLDST) and polyhistidine tags. Pro-KLks were purified from conditioned media using Ni\(^{2+}\)-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer’s instructions. After confirming the identity of purified proteins by Western blot analysis, pro-KLks were aliquoted and stored at −80°C.

**Molecular dynamics**

SFTI-FCQR variant/KLK4 complexes were generated by overlay of KLK4 (PDB ID 2BDG) and the trypsin/SFTI-1 complex (PDB ID 1SFI) in SPDBV v4.01 (RSMD 0.96 Å) [39] while mutations were made in YASARA Dynamics 9.12.13 [40]. Systems were solvated with TIP3P water and neutralized by Na\(^+\)/Cl\(^-\) counterions to a final concentration of 100 mM in VMD 1.8.7 [41]. This generated systems of approximately 28000 atoms, including 9000 water molecules.

Each protease–inhibitor complex was equilibrated using a stepwise relaxation procedure. In the first stage, all heavy-atoms were harmonically restrained with a force constant of 2 kcal/(mol Å\(^2\)) before a conjugate gradient minimization of 5000 steps was applied using NAMD 2.6 [38] and CHARMM27 force fields parameters. This was followed by heating to 298 K before simulating 500 ps under NPT conditions with periodic boundary conditions. A Langevin thermostat with a damping coefficient of 0.5 ps\(^{-1}\) was used to maintain the system temperature. The system pressure was maintained at 1 atm using a Langevin piston barostat. The particle mesh Ewald algorithm was used to compute long-range electrostatic interactions at every time step and non-bonded interactions were truncated smoothly between 7.5 Å and 9 Å. All covalent hydrogen bonds were constrained by the SHAKE algorithm or the SETTLE algorithm for water, permitting an integration time step of 2 fs. For the second stage, the restraints were retained on the protease and inhibitor α-carbons (C\(^{\alpha}\)) only, while all constraints were released in the third stage.

Three independent production runs of 5 ns were carried out for each system using ACEMD [37]. These simulations were performed under NVT with otherwise identical force field and simulation parameters as above. Coordinates were saved every 500 simulation steps producing 5000 frames per run. Analyses were performed using VMD 1.8.7 with hydrogen bond lengths and angles set to 40° and 3.3 Å respectively, chosen to align with the reported trypsin/SFTI-1 complex [17].

**Synthesis of SFTI variants**

All reagents were obtained from Auspep and all solvents from Merck unless stated otherwise. Inhibitors were synthesised as linear peptides on 2-chlorotrityl resin (1.3 mmol/g) derivatized with 0.9 mmol/g of the first residue, Ser (P1'). Coupling of the following nine residues was achieved using four-fold excess of Fmoc-protected amino acids dissolved in 0.25 M each of 2-[(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoropho-
spathe (HBTU), 1-hydroxybenzo-triazole (HOBr), and N,N-diisopropyl-2-(2-ethoxyvinyl)-amine (DIPEA) in N,N-Dimethylformamide (DMF). Fnoc protecting groups were removed by incubation in 10% piperidine and 5% 1,3-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF. The K features in the final four residues occurred as above, except that DMF was replaced with 'magic mix' solvent to prevent aggregation (Zhang et al., 1994). This contained equal parts of DMF, DCTh and N-methyl-2-pyrrolidone (NMP), or DMF, tolune and NMP for coupling and Fnoc removal respectively.

Linear peptides were liberated from the solid support by successive changes of 0.5% trifluoroacetic acid (TFA) in dichloromethane (DCM). Cyclisation of the peptide backbone was achieved in solution using 125 mM each of 1-hydroxy-7-azabenzotriazole (HOAt) and benzotriazol-1-yl-oxytrriphosphono- sphonium hexafluorophosphate (PyBOP) dissolved in DMF containing 0.25 M DIPA. Cyclization proceeded for 48 hr before dilution with an equal volume of DCM and extraction with H2O to remove residual reactants. Side chain protecting groups were removed from the dry product by cleavage for 2 hr in 95.75% TFA, with scavengers; 1.25% triisopropylsilane (TIS), 1.25% H2O and 3.75% triisooanisole. Cleaved peptides were purified from remaining synthetic by-products by reverse phase HPLC (rp-HPLC) across a gradient of 20-100% isopropanol using a Jupiter 4 µm Proteo 90A C-18 column (Phenomenex). Formation of the internal disulfide bond was achieved by overnight stirring in an aqueous redox buffer (150 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM reduced glutathione, 1 mM oxidised glutathione) while monitoring the reaction progress by MALDI-TOF mass spectrometry. Completed SFTI variants were purified, lyophilised and stored at −20°C.

Synthesis of peptide substrates

Peptide para-nitroaniline (pNA) substrates were synthesised on p-phenylenediamine (Sigma-Aldrich) derivatised 2-chlorotriyl resin (1.3 mmol/g) according to previously described methods [16,42]. Completed substrates were purified by rp-HPLC, validated by MALDI-TOF/MS and lyophilised before storage at −20°C.

Inhibition assays

Bovine β-trypsin, bovine α-chymotrypsin and human thrombin were obtained from Sigma while KLK12 and matriptase were from R&D Systems. Increasing concentrations of inhibitors were incubated with various concentrations of protease (final concentrations: KLK4, 1.5 nM; KLK12, 15 nM; KLK14, 2 nM; trypsin, 1 nM; matriptase, 4 nM; thrombin, 25 nM; α-chymotrypsin 25 nM) for 20 min in 200 µl assay buffer (100 mM Tris-HCl, 100 mM NaCl, 0.005% Triton-X, pH 8.0). Assays with thrombin and trypsin included 10 mM CaCl2. Enzyme activity was initiated by addition of substrate in 100 µl assay buffer (final concentration 100 µM; see Table 2). The rate of hydrolysis was measured at 405 nm over 7 min and was linear over this period. The extended assay period allowed for identification of inhibitors that were degraded. For SFTI-FCQR Asp14, Ki was determined by inhibition at various substrate concentrations using the competitive inhibition model and non-linear regression in Prism 5 (GraphPad Software Inc). The Ki for this inhibitor was also determined using the Morrison equation for tight binding inhibitors [43] [FVQR-pNA KiM = 679.2±113.1 µM [16]] and non-linear regression in Prism 5. Both methods produced comparable results (Table 2) and subsequent Ki values were determined with the Morrison method. Since SFTI-FCQR Asn14 had an IC50 below the concentration of KLK4, assays for this inhibitor were repeated with 0.15 nM KLK4 over 2 hr. The kcat and km values were determined from the lag phases and steady state of inhibition as previously described [44] using assay conditions as above and 500 µM substrate. Assays for inhibition of fibrinogen proteolysis used the same buffer and enzyme concentrations as above with 7 µM fibrinogen substrate. Proteolysis proceeded for 15 min (trypsin), 90 min (KLK4 and 14) or 180 min (KLK12) before termination by boiling in SDS-PAGE sample buffer. Proteolysis fragments were separated on 10% polyacrylamide gels.

Stability of SFTI variants in cell culture

The half-life in cell culture for SFTI-FCQR Asn14 and SFTI-FCQR Lys14 was determined using previously described methods [16]. Briefly, monolayers of LNCaP, 22Rv1 and PC3 cells were established in RPMI 1640 medium supplemented with 10% foetal calf serum (HyClone), 100 U/ml penicillin (Invitrogen) and 100 µg/ml streptomycin (Invitrogen). Cells were treated with ±1 µM inhibitor (SFTI-FCQR Asn14 or SFTI-FCQR Lys14) in fresh serum-containing media. Samples of media were taken at 24 hr intervals and boiled at 97°C for 15 min to denature serum protein. Residual inhibition by SFTI-FCQR variants was determined in competitive kinetic assays (as above), adding a volume of media to give 10 nM SFTI-FCQR Asn14 or 25 nM SFTI-FCQR Lys14 at 0 hr. Media without inhibitor was used to adjust for any endogenous media inhibition and data represent the mean ± SEM of three triplicate experiments.

Assessment of bioavailability in mice

Stability and bioavailability of SFTI-FCQR Asn14 in vivo was assessed in BALB/cFoxn1/Arc mice by oral, intravenous and intraperitoneal delivery (3 mg/kg). Inhibitor was dissolved in PBS at a concentration of 0.6 mg/ml prior to dosing. Serum levels of SFTI-FCQR Asn14 were subsequently measured by Liquid Chromatography-Mass Spectrometry (LC-MS) at Tetra Q laboratories (University of Queensland, Brisbane, Australia). This study was carried out in strict accordance to the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific purposes (7th edition 2004) and the protocol was approved by the University of Queensland Animal Ethics Committee (ABS group) which assigned the project approval code TetraQ/479/09/Bluebox. All efforts were made to minimize suffering by experimental animals.

Results

Molecular dynamics reveals a reduction in internal hydrogen bonds for SFTI-FCQR Asp14 compared to SFT1-1

The contribution of various SFT1-1 residues to inhibitor rigidity and complex stability was examined by molecular dynamics simulations on the trypsin/SFT1-1 complex (Figure 1A; PDB ID 1SFI). Post-simulation analysis of the internal hydrogen bond network agreed with the reported structure [17] regarding the reactive loop while differing in the side loop (Figure 1B). Most notably, rather than acting solely as a proton acceptor for the backbone amide of Gly1 and carbonyl oxygen of Phe12 seen in the crystal structure of SFT1-1 [21], it appeared that the Asp14-Arg2 side chain hydrogen bonds more often formed hydrogen bonds with the guanidino nitrogens of Arg2. These hydrogen bonds are evident in 20% of conformations often formed hydrogen bonds with the guanidino nitrogens of Arg2. These hydrogen bonds are evident in 20% of conformations.
simulation average structure as a reference indicated that although the change in conformation in the side loop varied from the SFTI-1 structure, the new conformation was stable (Figure 2B and C). The most rigid backbone atoms were found in residues that formed an extended $\beta$-sheet with trypsin (P3-P1) and as a result were flanked by both internal and intermolecular hydrogen bonds. Overall, the average number of internal hydrogen bonds for trypsin/SFTI-1 was 7.00 $\pm$ 0.07, equivalent to the total number identified in the crystal structure.

Previously, SFTI-1 was re-engineered to produce a selective KLK4 inhibitor (SFTI-FCQR Asp14) by optimising protease/inhibitor interactions [16]. To examine the impact of modifying the contact $\beta$-sheet of SFTI on the distribution of internal hydrogen bonds, corresponding simulations were performed on KLK4 (PDB ID 2BDG) in complex with a model of SFTI-FCQR Asp14. The resulting analysis suggested a marked reduction (mean = 3.70 $\pm$ 0.11) and rearrangement of the internal hydrogen bond network compared to SFTI-1 (Figure 2A). In the reactive loop, the side chain Thr4-Ser6 hydrogen bond was replaced by one between the carbonyl oxygen of Gln4 and the amide of Ser6, while Asp14 in the side loop no longer formed any recurrent internal hydrogen bonds. Perhaps as a result of the latter, the backbone hydrogen bonding pairs Phe2-Phe12 (Arg2-Phe12 in SFTI-1) and Gly7-Phe12 seen in the trypsin/SFTI-1 structure were again prevalent. These changes in internal hydrogen bonding pattern were accompanied by an altered conformation that poorly aligned with the SFTI-1 starting structure (Figure 2A) and reduced rigidity across the scaffold (Figure 2B and C).

Substitution at Asp14 alters internal hydrogen bonding in SFTI

Inspection of the KLK4/SFTI-FCQR Asp14 simulation trajectories revealed that Asp14 showed a high level of disorder and was too far from KLK4 to make contact and thus appeared not to contribute to complex stability. This suggested that substitution of Asp14 could present an opportunity to restore the internal hydrogen bonding network of the inhibitor. Structural imperatives restricted the opportunities for further replacements around the SFTI backbone and so no further substitutional analyses of these positions were undertaken. Accordingly, a library of SFTI-FCQR...

Figure 1. Representation of a trypsin/SFTI-1 complex and internal hydrogen bonding within SFTI variants during MD. Ribbon plot of SFTI-1 in complex with trypsin (A) with $\beta$-sheets and $\alpha$-helices coloured in yellow and blue respectively, excluding SFTI-1 which is displayed in magenta. The residues of the catalytic triad of trypsin and the P1 Lys of SFTI-1 are shown in stick models with carbon in green, nitrogen in blue and oxygen in red. The structure of SFTI variants are shown in ball and stick 2D model with intramolecular hydrogen bond networks for (B) SFTI-1, (C) SFTI-FCQR Asp14 and (D) SFTI-FCQR Asn14. Amino acids are labelled with one letter code and residue number in subscript while the frequency of hydrogen bonds per residue is in brackets (rounded to nearest tenth). Carbons, oxygen, nitrogen and sulphur are represented by gray, red, blue and yellow respectively while hydrogens are excluded for clarity. Bond lengths and angles are intentionally unrealistic to enable easy viewing of hydrogen bonds, represented by dotted green line. Only hydrogen bonds occurring in more than 50% of trajectory frames are shown. Data is represented as mean from three independent 5 ns MD trajectories.

doi:10.1371/journal.pone.0019302.g001
variants containing all naturally occurring amino acids (excluding cysteine) at residue 14 was simulated followed by hydrogen bonds analysis (Table 1). The frequency of internal hydrogen bonds in the starting structure (SFTI-FCQR Asp14) was only slightly above the median. Further, modifying residue 14 had a considerable effect on the internal hydrogen bond network across the nineteen SFTI-FCQR variants, ranging from 2.28 ± 0.07 (His14) to 4.29 ± 0.31 (Asn14) average hydrogen bonds. In contrast, these substitutions had little effect on the number of intermolecular hydrogen bonds, producing a slight decrease for the majority of variants. To verify these in silico results, six variants representative of the diverse residue side chains and the number of hydrogen bonds were synthesised: Asn14 (amide), Tyr14 (aromatic), Lys14 (basic), Gly14 (flexible), Ala14 (less flexible) and Ser14 (alcohol). These were screened against KLK4 in vitro to determine respective inhibition constants (Table 2).

SFTI-FCQR Asn14, predicted to have the most internal hydrogen bonds, was also the most potent KLK4 inhibitor with a $K_i$ of 0.0386 ± 0.0060 nM, exceeding that of SFTI-1 for trypsin ($K_i = 0.1$ nM). Furthermore, there was a consistent correlation between increasing number of internal hydrogen bonds during MD simulation and decreasing inhibition constants in vitro (Figure 3). However, it should be noted that SFTI-FCQR Lys14 assays were carried out immediately after addition of inhibitor since this variant was degraded after prolonged incubation with KLK4 ($t_{1/2} = 5.63 ± 6.2$ minutes). This may reflect the introduction of a second potential cut site for trypsin-like proteases (Lys) on the side loop of SFTI. Although the Arg5-Ser6 scissile bond can be cleaved on SFTI without detrimental effect, the side loop does not have the features of a canonical loop and cleavage of the Gly1-Lys14 peptide bond may be irreversible.

Examining the internal hydrogen bonding network of SFTI-FCQR Asn14 revealed that substitutions at position 14 influenced their frequency and distribution across the entire scaffold (Figure 1C). In the side loop, the Asn14 side chain formed a $K_i$ of 0.0386 ± 0.0060 nM, exceeding that of SFTI-1 for trypsin ($K_i = 0.1$ nM). Furthermore, there was a consistent correlation between increasing number of internal hydrogen bonds during MD simulation and decreasing inhibition constants in vitro (Figure 3). However, it should be noted that SFTI-FCQR Lys14 assays were carried out immediately after addition of inhibitor since this variant was degraded after prolonged incubation with KLK4 ($t_{1/2} = 5.63 ± 6.2$ minutes). This may reflect the introduction of a second potential cut site for trypsin-like proteases (Lys) on the side loop of SFTI. Although the Arg5-Ser6 scissile bond can be cleaved on SFTI without detrimental effect, the side loop does not have the features of a canonical loop and cleavage of the Gly1-Lys14 peptide bond may be irreversible.

Examining the internal hydrogen bonding network of SFTI-FCQR Asn14 revealed that substitutions at position 14 influenced their frequency and distribution across the entire scaffold (Figure 1C). In the side loop, the Asn14 side chain formed...
### Table 1. *In silico* Internal Hydrogen Bond Analysis of SFTI-FCQR Residue 14 Variants.

| SFTI variant | Internal Hydrogen Bonds (Mean ± SEM) | % Change from SFTI-FCQR Asp<sub>14</sub> | Intermolecular Hydrogen Bonds (Mean ± SEM) | % Change from SFTI-FCQR Asp<sub>14</sub> |
|--------------|--------------------------------------|------------------------------------------|--------------------------------------------|------------------------------------------|
| SFTI-FCQR Asn<sub>14</sub> | 4.68 ± 0.086 | 26.5 | 8.46 ± 0.10 | 2.1 |
| SFTI-FCQR Val<sub>14</sub> | 4.26 ± 0.14 | 15.1 | 8.17 ± 0.18 | 0.1 |
| SFTI-FCQR Tyr<sub>14</sub> | 4.07 ± 0.24 | 9.9 | 8.22 ± 0.30 | -0.7 |
| SFTI-FCQR Met<sub>14</sub> | 3.98 ± 0.12 | 7.6 | 8.11 ± 0.20 | -2.0 |
| SFTI-FCQR Lys<sub>14</sub> | 3.91 ± 0.47 | 5.6 | 8.11 ± 0.17 | -2.1 |
| SFTI-FCQR Phe<sub>14</sub> | 3.89 ± 0.15 | 5.1 | 7.58 ± 0.08 | -8.5 |
| SFTI-FCQR Ile<sub>14</sub> | 3.85 ± 0.43 | 4.2 | 7.30 ± 0.35 | -11.8 |
| SFTI-FCQR Asp<sub>14</sub> | 3.70 ± 0.11 | 0 | 8.28 ± 0.19 | 0 |
| SFTI-FCQR Gly<sub>14</sub> | 3.67 ± 0.30 | -0.9 | 7.86 ± 0.17 | -5.1 |
| SFTI-FCQR Pro<sub>14</sub> | 3.66 ± 0.43 | -1.0 | 6.49 ± 0.23 | -21.7 |
| SFTI-FCQR Glu<sub>14</sub> | 3.59 ± 0.50 | -3.0 | 7.83 ± 0.36 | -5.5 |
| SFTI-FCQR Arg<sub>14</sub> | 3.51 ± 0.51 | -5.1 | 7.65 ± 0.04 | -7.6 |
| SFTI-FCQR Gln<sub>14</sub> | 3.36 ± 0.25 | -9.2 | 7.52 ± 0.14 | -9.2 |
| SFTI-FCQR Leu<sub>14</sub> | 3.33 ± 0.26 | -10.0 | 8.11 ± 0.17 | -7.7 |
| SFTI-FCQR Trp<sub>14</sub> | 3.12 ± 0.22 | -15.6 | 7.92 ± 0.36 | -4.3 |
| SFTI-FCQR Thr<sub>14</sub> | 2.93 ± 0.093 | -21.0 | 7.34 ± 0.06 | -11.3 |
| SFTI-FCQR Ala<sub>14</sub> | 2.89 ± 0.085 | -21.8 | 7.25 ± 0.16 | -12.4 |
| SFTI-FCQR Ser<sub>14</sub> | 2.56 ± 0.048 | -30.8 | 7.52 ± 0.10 | -9.1 |
| SFTI-FCQR His<sub>14</sub> | 2.28 ± 0.068 | -38.2 | 8.07 ± 0.11 | -2.5 |

### Table 2. Inhibitory Properties of SFTI-1, SFTI-FCQR and SFTI-FCQR Residue 14 Variants.

| Enzyme | Inhibitor | IC<sub>50</sub> (nM) | \( K_i \) (nM) | Morrison \( K_i \) (nM) | Theoretical Mass | Determined Mass | Substrate (100 μM) |
|--------|-----------|----------------------|----------------|--------------------------|-----------------|-----------------|------------------|
| KLK4   | SFTI-FCQR Asn<sub>14</sub> | 0.0635 ± 0.0024 | - | 0.0386 ± 0.0060 | 1559.75 | 1560.40 | FVQRpNA |
|        | SFTI-FCQR Tyr<sub>14</sub> | 3.47 ± 0.20 | - | 2.55 ± 0.43 | 1610.77 | 1610.56 | |
|        | SFTI-FCQR Lys<sub>14</sub> | 6.07 ± 0.13 | - | 3.56 ± 0.27 | 1573.80 | 1574.93 | |
|        | SFTI-FCQR Asp<sub>14</sub> | 7.97 ± 1.08 [16] | 3.62 ± 0.26 | 3.89 ± 0.40 | 1560.73 | 1559.94 | |
|        | SFTI-FCQR Gly<sub>14</sub> | 14.74 ± 1.089 | - | 10.39 ± 2.87 | 1502.73 | 1504.77 | |
|        | SFTI-FCQR Ala<sub>14</sub> | 26.23 ± 0.85 | - | 18.31 ± 3.36 | 1516.74 | 1517.99 | |
|        | SFTI-FCQR Ser<sub>14</sub> | 29.23 ± 1.081 | - | 21.24 ± 3.81 | 1532.74 | 1533.94 | |
|        | SFTI-1 | 221 ± 10.1 [16] | - | - | 1514.75 | 1514.84 | |
| KLK14  | SFTI-FCQR Asp<sub>14</sub> | 1506 ± 37.1 [16] | - | - | - | - | Ac-GLSR-pNA |
|        | SFTI-FCQR Asn<sub>14</sub> | 251 ± 21.9 | - | - | - | - | |
| β-Trypsin | SFTI-FCQR Asp<sub>14</sub> | 4064 ± 109 [16] | - | - | - | - | BAPNA |
|        | SFTI-FCQR Asn<sub>14</sub> | 2178 ± 145 | - | - | - | - | |
|        | SFTI-1 | - | 0.1 [17] | - | - | - | |
| Matriptase | SFTI-FCQR Asn<sub>14</sub> | >10,000 | - | - | - | - | Bz-FVIRpNA |
|        | SFTI-1 | - | 0.92 [19] | - | - | - | N-t-Boc-QAR-AMC |
| Thrombin | SFTI-FCQR Asn<sub>14</sub> | >10,000 | - | - | - | - | Bz-FVIRpNA |
|        | SFTI-1 | - | 5050 [19] | - | - | - | N-t-Boc-LIRR-AMC |
| Chymotrypsin | SFTI-FCQR Asn<sub>14</sub> | >10,000 | - | - | - | - | WpNA |
|        | SFTI-1 | - | 2300 ± 100 [51] | - | - | - | N-succinyl-AAPPpNA |

Amino acids are represented by the one letter code.

PLOS ONE | www.plosone.org 6 April 2011 | Volume 6 | Issue 4 | e19302
hydrogen bonds with the backbone amides of Phe2 and Gly1 with similar prevalence as seen for corresponding residues in SFTI-1. In comparison to SFTI-FCQR Asp14, the hydrogen bonds between Phe2-Phe12 and Gly1-Phe12 were similarly frequent while a further hydrogen bond was prevalent between the backbone amide of Asn14 and the carbonyl oxygen of Phe12. Overall, it appeared that changes in the hydrogen bonding pattern in the side loop of SFTI-FCQR Asn14 restored the frequency of the hydrogen bonds in the reactive loop to the level determined for SFTI-1. A previous study also reported that hydrogen bonds of the side loop, in particular the one formed between carboxylic oxygen of Asp14 and the Gly2 amide, were necessary to provide rigidity to the SFTI-1 reactive loop [20]. Consequently, this resulted in a reactive loop that closely aligned with the SFTI-1 starting structure both in terms of conformation (Figure 2A) and structural stability (Figure 2B and C). Consistent with a highly rigid scaffold, \( k_{\text{off}} \) was found to be 0.031 ± 0.010 s$^{-1}$ with a calculated second order rate constant \( (k_{\text{off}}/K_{i}) \) of 8.03 ± 10$^8$ M$^{-1}$ s$^{-1}$ (Figure 4), suggesting that SFTI-FCQR Asn14 binding to KLK4 is diffusion controlled. Collectively, these findings indicate that residue 14 of the side loop is instrumental for maintaining conformational stability of the SFTI reactive loop, a requirement for potent standard mechanism inhibition.

SFTI-FCQR Asn14 is a selective KLK4 inhibitor

Screening SFTI-FCQR Asn14 against a panel of serine protease targets revealed that this variant was more selective than SFTI-FCQR Asp14. The most closely related enzyme to KLK4 is KLK14 with 85% sequence identity within 5 Å of the catalytic triad, while trypsin is a high affinity target for SFTI-1. Although SFTI-FCQR Asn14 more potently inhibited KLK14 and trypsin, the relative increase in inhibition was only six-fold and two-fold respectively, compared to 125-fold improvement for KLK4 (Table 2). This likely reflects that increasing hydrogen bonds, and therefore binding loop rigidity, produces a more potent inhibitor in general. Matriptase, thrombin and 3-chymotrypsin which are also inhibited by SFTI-1, showed no inhibition with SFTI-FCQR Asn14 at 10,000 nM.

Further, it has been demonstrated that amidolytic inhibition of a small peptide substrate does not necessarily equate to proteolytic inhibition. For example, SFTI-1 inhibits KLK4 in amidolytic assays (IC$_{50}$ = 221 ± 10.1 nM) but not in fibrinogen digestion assays with 2 μM inhibitor [16]. Consequently, the ability of
SFTI-FCQR Asn₁₄ to inhibit proteolysis of fibrinogen by KLK4, KLK12, KLK14 and trypsin was assessed. KLK4 proteolysis was blocked at inhibitor concentrations as low as 62.5 nM for SFTI-FCQR Asn₁₄ compared to 250 nM for SFTI-FCQR Asp₁₄, with more robust inhibition of degradation of the KLK4-preferred fibrinogen α-chain (Figure 5A–B). No inhibition of KLK12, KLK14 or trypsin fibrinogen proteolysis by SFTI-FCQR Asn₁₄ occurred up to 10,000 nM (Figure 5C–F).

Figure 5. Selective inhibition of serine protease proteolytic activity by SFTI-FCQR Asn₁₄. Examination of fibrinogen proteolysis by trypsin and kallikreins by SDS-PAGE. Bands were visualised with Coomassie blue staining after resolving on 10% polyacrylamide gels. Images are representative of three separate experiments. Inhibition of KLK4 proteolytic activity by (A) SFTI-FCQR Asp₁₄ and (B) SFTI-FCQR Asn₁₄. Inhibition of trypsin proteolytic activity by (C) SFTI-1 and (D) SFTI-FCQR Asn₁₄. Inhibition of proteolytic activity of (F) KLK12 and (F) KLK14 by SFTI-FCQR Asn₁₄. doi:10.1371/journal.pone.0019302.g005
SFTI-FCQR Asn₁₄ is stable in culture with prostate cancer cells

Previous evaluation of SFTI-FCQR Asp₁₄ stability in culture with prostate cancer cells revealed that it was highly resistant to breakdown [16]. Whether replacing Asp₁₄ with Asn markedly altered inhibitor stability in a cellular environment was assessed by calculating the half-life of SFTI-FCQR Asn₁₄. Additionally, the SFTI-FCQR Lys₁₄ half-life was determined given that this variant seemed to be degraded in competitive kinetic assays (see above). For SFTI-FCQR Asn₁₄, inhibition of KLK₄ gradually declined over time indicating a slow rate of decay that was comparable across each cell line (figure 6A). Despite an average reduction in stability compared to SFTI-FCQR Asp₁₄, a half-life 55–70 hours is still well above the expected clearance time for peptide-based therapeutics in vivo. Further, in agreement with previous observations, SFTI-FCQR Lys₁₄ was rapidly degraded with two-thirds of the initial activity lost within the first 24 hr (figure 6B).

SFTI-FCQR Asn₁₄ is bioavailable in mice when administered by intraperitoneal perfusion

While other BBIs are readily bioavailable [45] whether this applies to SFTI-1 or previously produced variants is yet to be determined. To establish the pharmacokinetic profile of SFTI-FCQR Asn₁₄, the inhibitor was delivered via intravenous (IV), intraperitoneal (IP) and oral routes to BALB/c mice before serum samples taken over time were quantified by LC-MS. Orally delivered SFTI-FCQR Asn₁₄ did not result in detectable levels of inhibitor in serum. In contrast IV and IP administered SFTI-FCQR Asn₁₄ at a dosage of 3 mg/kg had a serum half-life of 25–28 mins with a residual inhibition concentration of 10.0±0.8 nM after 4 hours irrespective of delivery route (Figure 7).

Discussion

This study has shown that when re-engineering a canonical serine protease inhibitor preserving binding loop rigidity and conformation is essential to maintain high binding affinity. Indeed, inhibitor variants with more frequent internal hydrogen bonds in silico correlated with more potent inhibition in vitro, emphasising their role in tight binding complexes. This guided production of an inhibitor with 125-fold improved potency for KLK₄ and enhanced selectivity over off-target proteases, including closely related KLKs. Further, SFTI-FCQR Asn₁₄ was stable in a cancer cell milieu and bioavailable by intraperitoneal perfusion in mice, making it an attractive candidate for further therapeutic development.

The suitability of SFTI as a generic scaffold for inhibitor design and its properties for maintaining structural integrity within a cell environment have previously been discussed in detail [4,16]. Although SFTI-FCQR Asn₁₄ was less stable than SFTI-FCQR Asp₁₄ in culture with prostate cancer cells, it was sufficiently resistant to degradation, highlighting the robustness of the SFTI scaffold. The fact that SFTI-FCQR Asn₁₄ was also bioavailable by IP indicates that the inhibitor had the ability to diffuse across tissues in vivo. This suggests that using a slow release depot implant is a viable mode of delivery. Alternatively there are numerous methods available for improving the retention of peptide drugs as previously reviewed [4]. Most notably, MD analysis indicated that Ile₁₀ did not make contact with KLK₄ and is positioned to provide an anchoring point for PEGylation [46] unlikely to markedly affect inhibitory properties.
Producing potent and selective standard mechanism inhibitors depends on fully realizing the highly conserved and successful structural features of the canonical loop. These inhibitors show two important properties. First, their reactive sites occur in constrained binding loops with similar structure and conformation [25]. Second, rigidity is maintained by intrinsic structural determinants allowing for positioning the P1′ free amine for peptide bond reformation after the scissile bond is cleaved [30,31]. Structural comparison delineates 19 families of inhibitors (I1-I3, I7, I8, 110-13, 115-20, I36 and I40) that belong to 13 clans comprising distinct protein folds and evolutionary origins [25].

The fact that the conformation of the canonical binding loop has evolved numerous times independently highlights its versatility as a starting structure for inhibitor design. However, whilst most tight binding standard mechanism inhibitors with large, flexible contact surfaces are slow binding, the SFTI scaffold is reduced to a simple canonical loop allowing for both fast and tight binding.

Strategies to re-engineer serine protease inhibitors commonly focus on the active site binding β-sheet of the canonical loop. The previous production of a selective KLK4 inhibitor utilised this approach by grafting a preferred substrate sequence into the β-sheet of SFTI-1 [16]. While the importance of canonical loop rigidity is well appreciated in naturally occurring structures, it has not received similar attention when modifying these inhibitors for new targets. The present study focused on restoring the internal hydrogen bonding network of the SFTI scaffold, generating an inhibitor with considerably increased affinity. Indeed, modifications of residue 14 that enhanced internal hydrogen bonding increased potency of inhibition across all variants assayed in vitro. This may also explain the modest increase in affinity for trypsin and KLK14 by SFTI-FCQR Asn14. Consistent with the importance of residue 14 to hydrogen bonding, a previous study found that substituting Asp14 for Ala in SFTI-1 resulted in a marked reduction in potency for trypsin [47]. Thus two complementary strategies for enhancing inhibitor performance are evident; re-engineering the active site binding β-sheet of SFTI is instrumental in achieving selectivity while modulating the internal hydrogen bonding network engenders increased potency. Bringing these two components together in SFTI-FCQR Asn14 resulted in an inhibitor rivalling SFTI-1 in terms of potency without its promiscuity.

The findings presented here are in agreement with a previous in silico study on SFTI-1 using graph representation [48] to analyse how various internal hydrogen bonds contribute to structural rigidity [20]. Similarly, it was observed that the hydrogen bonds within the side loop were important for maintaining rigidity of the SFTI-1 reactive loop. The strongest contribution was conferred by the hydrogen bonding pairs of Asp14-Gly1 followed by Phe12-Gly1 and Phe12-Arg2. Although the findings from both studies closely align, graph representation is confined to structurally determined hydrogen bonding patterns. As a result, hydrogen bonds are either present or absent, preventing prediction of how subtle changes in these patterns and frequencies will affect binding affinities.

The prominent role of internal hydrogen bonding in maintaining canonical loop rigidity is not only limited to the SFTI scaffold. The same study by Costa and co-workers showed that hydrogen bonds are vital for stabilising the binding loop for BBIIs (Family I12) in general [20]. Further, studies on Eglin C (Family I13) assessed point mutations by NMR and competitive binding assays, highlighting the relationship between inhibition constants and hydrogen bonds within the binding loop [49]. In fact the importance of the internal hydrogen bonding network in maintaining the conformation of the canonical loop has been demonstrated structurally across most families of standard mechanism inhibitors [50]. Preservation of these interactions is a key, yet often overlooked, property to consider when re-engineering this class of inhibitors.

In contrast to small-molecule inhibitors with few rotational bonds, conventional docking and scoring of protein-based inhibitors and their receptors have markedly lower rates of success [32]. The alternative is to calculate average binding affinities across MD trajectories including conformations of many low energy-state complexes [32]. These methods fail to recapitulate the most important aspect of standard mechanism serine protease inhibitors: the conformation and rigidity of the canonical loop before and after cleavage of the scissile bond as well as during the acyl-enzyme intermediate. Therefore, it is more effective to use computer-aided design methods to optimize structural features supporting the binding loop conformation to more closely replicate the native starting structure. This study focused on analysing one of these intrinsic canonical loop properties, namely hydrogen bonds, to accurately predict relative binding affinities of SFTI variants. We suggest that this simple approach is generally applicable as a tool to enhance binding affinity when re-engineering standard mechanism inhibitors. Used in conjunction with traditional peptidic library screens to sample protease substrate preferences, this strategy is likely to produce both highly selective and potent inhibitors.

Author Contributions

Conceived and designed the experiments: JES SJdV CFR JMH. Performed the experiments: JES SJdV KCS. Analyzed the data: JES SJdV JMH. Contributed reagents/materials/analysis tools: AMB JMH. Wrote the paper: JES SJdV AMB JMH.

References

1. Jemal A, Siegel R, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60: 277–300.
2. Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, et al. (1999) Natural history of progression after PSA elevation following radical prostatectomy. JAMA 281: 1591–1597.
3. Soitoopoulos G, Pampalakis G, Diamandis EP (2009) Functional roles of human kallikrein-relevant peptides. J Biol Chem 284: 32899–32994.
4. Swedberg JE, de Vree SJ, Harris JM (2010) Natural and engineered kallikrein inhibitors: an emerging pharmacopeia. Biol Chem 391: 357–374.
5. Goerig P, Magdonov V, Brandtsteder H (2010) Natural and synthetic inhibitors of kallikrein-related peptidases (KLKs). Biochimie, (In press) DOI: 10.1016/j.bioch.2010.06.022.
6. Lawrence MG, Lai J, Clements JA (2010) Kallikrein on steroids: structure, function, and hormonal regulation of prostate-specific antigen and the extended extended loop. J Biol Chem 383: 405–413.
7. Xi Z, Klokst TI, Korkmaz K, Kurns P, Elsh C, et al. (2004) Kallikrein 4 is a Prominently Nuclear Protein and Is Overexpressed in Prostate Cancer. Cancer Res 64: 2563–2570.
8. Veveris-Lowe TL, Lawrence MG, Collard RL, Bui L, Herington AC, et al. (2005) Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. Endocr Relat Cancer 12: 631–643.
9. Obiezu CV, Michael IP, Levesque MA, Diamandis EP (2006) Human kallikrein 12 proteinases of the prostate-specific serine protease KLK4 defined by positional-scanning peptide libraries. Prostate 62: 1–13.
10. Beaumont N, Debeia M, Greutzhut S, Kellermann J, Bode W, et al. (2006) Interplay of human tissue kallikrein 4 (hK4) with the plasminogen activation system: hK4 regulates the structure and functions of the urokinase-type plasminogen activator receptor (uPAR). Biol Chem 387: 217–222.
11. Mize GJ, Wang W, Takayama TK (2008) Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through prostate-activated receptors-1 and -2. Mol Cancer Res 6: 1043–1051.
Serine Protease Inhibitor Engineering

13. Greenberg DL, Mize GJ, Takayama TK (2003) Protease-activated receptor mediated RhoA signaling and cytoskeletal reorganization in LNCaP cells. Biochemistry 42: 702–709.

14. Ramsay AJ, Dong Y, Hunt ML, Linu M, Samaratunga H, et al. (2008) Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. J Biol Chem 283: 12293–12304.

15. Gao J, Collard RL, Bui L, Herington AC, Nicol DL, et al. (2007) Kallikrein 4 is a potential mediator of cellular interactions between cancer cells and osteoblasts in metastatic prostate cancer. Prostate 67: 348–360.

16. Swedberg JE, Nigon LV, Reid JC, de Veer SJ, Walpole CM, et al. (2009) Solution structures by 1H NMR of the novel cyclic trypsin inhibitor SFTI-1 from sunflower seeds. J Mol Biol 390: 525–533.

17. Laskowski Jr. M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

18. Laskowski M, Qasim MA (2000) How to reconstruct enzyme-inhibitor complexes from the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

19. Laskowski Jr. M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

20. Costa JR, Yaliraki SN (2006) Role of rigidity on the activity of proteinase inhibitors. Adv Enzymol Relat Areas Mol Biol 61: 201–301.

21. Korsinczky ML, Schirra HJ, Rosengren KJ, West J, Condie BA, et al. (2001) The slow, tight-binding inhibition of cathepsin B by leupeptin. A hysteretic effect. Eur J Biochem 278: 21702–21708.

22. Hubbard SJ, Campbell SF, Thornton JM (1991) Molecular recognition. Adv Enzymol Relat Areas Mol Biol 61: 201–301.

23. Madala PK, Tyndall JD, Nall T, Fairlie DP (2010) Update 1 of: Protease inhibitors Universally Recognize Beta Strands In Their Active Sites. Chem Rev. DOI: 10.1021/cr900368a.

24. Marx UC, Korsinczky ML, Schirra HJ, Jones A, Condie B, et al. (2003) Enzymatic cyclization of a potent bowerman-birk protease inhibitor, sunflower trypsin inhibitor-1, and solution structure of an acyclic precursor peptide. J Biol Chem 278: 21702–21708.

25. Rawlings ND, Tolle DP, Barrett AJ (2004) Evolutionary families of peptidase inhibitors. Biochem J 378: 705–716.

26. Laskowski Jr. M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

27. Laskowski Jr. M, Qasim MA (2000) How to reconstruct enzyme-inhibitor complexes from the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

28. Laskowski Jr. M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochim Biophys Acta 1477: 324–337.

29. Shaw GL, Davis B, Keeler J, Fersht AR (1995) Backbone dynamics of peptidase inhibitors of serine proteases. Cell Mol Life Sci 60: 8755–8766.

30. Ramsay AJ, Dong Y, Hunt ML, Linu M, Samaratunga H, et al. (2008) Substrate-guided design of a potent and selective kallikrein-related peptidase inhibitor for kallikrein 4. Chem Biol 15: 219–234.

31. Zukharova E, Horvath MP, Goldenberg DP (2009) Structure of a serine protease poised to re-synthesize a peptide bond. Proc Natl Acad Sci U S A 106: 11034–11039.

32. Sousa SF, Fernandes PA, Ramos MJ (2006) Protein-ligand docking: current status and future challenges. Proteins 65: 15–26.

33. Anderson JA, Lyubimov A, Tracey K, Cooper JA, et al. (2005) Scalable molecular dynamics simulations fully implemented on graphics processing units. J Comput Phys 227: 3534–3539.

34. Harvey M, Giuseppe G, De Fabritius G (2009) ACME: Accelerated molecular dynamics simulations in the microseconds timescale. J Chem Theory Comput 51: 1632.

35. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, et al. (2005) Scalable molecular dynamics with NAMD. J Comput Chem 26: 1781–1800.

36. Abbenante G, Leung D, Bond T, Fairlie DP (2000) An efficient Fmoc strategy for the rapid synthesis of peptide para-nitroanilides. Lett Pept Sci 7: 347–351.

37. Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14: 33–38,27–38.

38. Madala PK, Tyndall JD, Nall T, Fairlie DP (2010) Update 1 of: Protease inhibitors Universally Recognize Beta Strands In Their Active Sites. Chem Rev. DOI: 10.1021/cr900368a.

39. Morrison JF, Walsh CT (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling. Electrophoresis 18: 2714–2723.

40. Madala PK, Tyndall JD, Nall T, Fairlie DP (2010) Update 1 of: Protease inhibitors Universally Recognize Beta Strands In Their Active Sites. Chem Rev. DOI: 10.1021/cr900368a.