Isolation of High-Affinity Trypsin Inhibitors from a DNA-Encoded Chemical Library

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Experimental Section

Synthetic protocols

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich-Fluka (Buchs, Switzerland). The procedures for the synthesis of the DNA-encoded library of 620 compounds have been described elsewhere [15]. In short, 48-mer oligonucleotides (IBA GmbH, Göttingen, Germany), carrying a free amino group at the 5’ end (ω-aminohexyl phosphate diester) were used, with general structure: 5’-GGA GCT TCT GAA TTC TGT GTG CTG XXX XXX CGA GTC CCA TGG CGC AGC-3’, where the XXX XXX coding sequence unambiguously identifies the individual compounds. Chemical compounds, carrying carboxylic acid, anhydride, N-hydroxysuccinimide ester or isothiocyanate groups, were coupled to the primary amino group of the oligonucleotides. The synthesis of bidentate conjugates of benzamidine and compound 73 is shown in Supplementary Figure 2. The synthesis and analytical data for the two most potent bidentate trypsin inhibitors were as follows:

\( N-(4\text{-carbamimidoylbenzyl})-5-(3\text{-}(3\text{-iodophenyl})\text{thioureido})\text{pentanamide (8)} \): 3-Iodophenyl isothiocyanate (23 mg, 88 \( \mu \)mol) was dissolved in 1 ml DMSO, added to 5-Amino-n-valeric acid (10mg, 88 \( \mu \)mol) in 1 ml DMSO/H\(_2\)O. 100 \( \mu \)L of 0.5 M NEt\(_3\) pH 9.9 was added, and the mixture was stirred over night at 30°C. The resulting conjugate was purified by HPLC, dried, and redissolved in 0.5 ml DMSO. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (180 \( \mu \)mol) and N-hydroxy succinimide (180 \( \mu \)mol), dissolved in DMSO, were added, and the mixture was stirred for 30 minutes at 30°C. After addition of 4-Aminomethyl-benzamidine (2) (180 \( \mu \)mol in 1 ml DMSO), the mixture was stirred over night at 30°C, and quenched with a molar excess of propylamine. The reaction product was purified by HPLC. 

\(^1\)H NMR (DMSO-d6, 400 MHz) \( \delta \) 1.56 (m, 4H), 2.21 (d, 2H), 3.46 (m, 2H), 4.36 (d, 2H), 7.10 (m, 1H), 7.42 (m, 4H), 7.75 (m, 2H), 7.94 (s, 2H), 8.47 (m, 1H), 8.90 (s, 2H), 9.25 (s, 2H). ESI-MS calculated for [M+1]\(^+\): 510.07. Found: 510.13.

\( N-(4\text{-carbamimidoylbenzyl})-2-(3\text{-}(3\text{-iodophenyl})\text{thioureido})\text{phenyl}acetamide (10) \) Synthesis same as for 8, with the difference that 3-Aminophenylacetic acid was used as a bifunctional linker instead of 5-Amino-n-valeric acid. \(^1\)H NMR (DMSO-d6, 400 MHz) \( \delta \) 3.46 (s, 2H), 4.32 (d, 2H), 7.06 (m, 2H), 7.24 (m, 1H), 7.38 (m, 6H), 7.69 (m, 2H), 7.92 (m, 1H), 8.64 (m, 1H), 8.92 (s, 1H), 9.21 (s, 2H), 9.90 (s, 1H), 10.00 (s, 1H). ESI-MS calculated for [M+1]\(^+\): 544.06. Found: 544.12.
Trypsin coating of sepharose beads
CNBr-activated sepharose (GE Healthcare, Piscataway, NJ) was swollen in 1 mM HCl, washed, and mixed in separate tubes with 2.5, 0.5, 0.1 and 0.02 mg/ml bovine pancreatic trypsin (Sigma-Aldrich-Fluka, Buchs, Switzerland) dissolved in 0.1 M Tris-Cl, 0.5 M NaCl, pH 8.3. After 4 h incubation at 4°C, the slurry was repeatedly washed and stored in 0.1 M NaAc, 0.5 M NaCl, pH 4 at 4°C.

Microarray construction
For microarray decoding, 19-mer 5’ amino-tagged oligonucleotides (Sigma-Genosys, Steinheim, Germany) with general sequence 5’-T GTG CTG XXX XXX CGA GTC-3’ (XXX XXX representing the 6 nucleotide code) were spotted in quadruplicate [1 nl of a 20 µM oligonucleotide solution in 3xSSC (0.45 M NaCl, 45 mM Na-citrate, pH 7.0)] onto 25 x 75 mm epoxy-activated microarray slides (Functional Genomics Center Zurich, Switzerland), using a Flexarrayer robot (Scienion, Berlin, Germany). After spotting, slides were incubated in a humid chamber overnight at room temperature.

Selection procedures
The oligonucleotide 5’-GGA GCT TCT GAA TTC TGT GTG CTG-3’ was radioactively labeled at the 5’ terminus using γ33P-ATP (GE Healthcare) and T4 polynucleotide kinase (USB, Cleveland, OH). The radiolabeled oligonucleotide was mixed with the benzamidine modified oligonucleotide (see Supplementary figure 1) at 45 nM concentration, and hybridization was allowed by incubating for 10 minutes at 50°C. In separate tubes, aliquots of the oligonucleotide fragments were mixed with 50 µL of trypsin-coated sepharose slurry of different coating densities, which had previously been pre-blocked in 0.2 mg/ml herring sperm DNA (Novagen, Madison, WI). After repeated rounds of washing in SpinX columns (Corning Life Sciences, Acton, MA), the remaining beads as well as aliquots of the input, flow-through and all washing fractions were subjected to 33P radioactivity counting with a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Library selections were performed essentially as described[9]. In short, the above mentioned benzamidine-modified oligonucleotide was mixed and hybridized with the previously described library of 620 DNA-encoded compounds[15]. Selections with the resulting double stranded library were performed with trypsin-coated sepharose slurry (0.1 and 0.02 mg/ml coating density) and with sepharose slurry without coated protein as described above. For decoding, aliquots of the library and the samples after selection were used as templates in a 2-step PCR reaction (1st step: 25 cycles of 1 min at 94 °C; 1 min at 55 °C; 20 s at 72 °C) using primers ABfo_short (5’-GGA GCT TCT GAA TTC TGT GTG CTG-3’; 400 nM) and Elib2ba (5’-GCT GCG CCA TGG GAC TCG-3’; 400 nM; IBA) in the first step – and only primer Cy3 labeled primer Elib2ba (400 nM; IBA) in the 2nd step with purified PCR products of 1st step PCR reaction as templates (22 cycles of 1 min at 94 °C; 1 min at 55 °C; 20 s at 72 °C),
yielding an excess of Cy3-labeled single-stranded target DNA. The reaction product was extracted with phenol / chloroform using Phase Lock Gel tubes (Eppendorf, Hamburg, Germany) and precipitated in ethanol. The DNA pellet was resuspended in 100 µl hybridization buffer (4x SSC, 50 mM HEPES, 0.2% (w / v) SDS, pH 7) and incubated with the above described oligonucleotide microarrays in a Tecan HS400 hybridization instrument (Tecan, Männedorf, Switzerland) for 4 h at 44 °C; this was followed by successive washing steps with 2x SSC / 0.2% (w / v) SDS, 0.2x SSC/0.2% (w / v) SDS and 0.2x SSC for 90 s. After hybridization, microarrays were analyzed using a GenePix Professional 4200A instrument (Molecular Devices, Sunnyvale, CA; λex = 635 nm; 100% laser power; gain of photomultiplier = 350). Spot intensities were quantified using GeneSpotter image analysis software (MicroDiscovery). The local background was subtracted from the mean value of each spot, and the average of 4 spot values gave the signal value for each compound.

**Protease inhibition assays**

The concentration of inhibitors in solution was determined by NMR, using nitromethane as internal standard. In the trypsin inhibition assays, 2 nM of bovine pancreatic trypsin (Sigma) in PBS (20 mM NaH2PO4, 30 mM Na2HPO4, 100 mM NaCl, pH 7.4) was incubated with varying concentrations of the inhibitors in 96-well microtiter plates (Nunc, Roskilde, Denmark) for 15 minutes at room temperature. The reaction was started by addition of the fluorogenic substrate Z-RRG-AMC (Bachem, Bubendorf, Switzerland) dissolved in PBS to an end concentration of 0.1 mM in a total volume of 100 µL. The change of fluorescence signal (ex: 383 nm; em: 455 nm; cutoff filter: 420 nm) was recorded over 10 minutes using a SpectraMax microplate reader (Molecular Devices). The rate of fluorescence signal increase over time (reaction velocity v) was plotted against the corresponding inhibitor concentrations, and the IC50 value for the inhibitor was obtained by fitting to the equation below [Eq1] (KaleidaGraph V 4.0, Synergy Software; [I]0: initial inhibitor concentration).

$$v = \frac{v_{\text{max}}}{1 + \frac{[I]_0}{IC_{50}}}$$

The inhibition assays for Factor Xa (from bovine plasma, Sigma) was performed in Factor Xa reaction buffer (50 mM Tris-Cl, 100 mM NaCl, 5mM CaCl2, pH 8) and for Thrombin (from bovine plasma, Sigma) in Thrombin reaction buffer (20 mM Tris-Cl, 150 mM NaCl, 2.5mM CaCl2, pH 8.4), with an enzyme concentration of 0.5 µg/ml for both proteases and with a substrate concentration of 0.2 mM.
Supplementary schemes and figures

Supplementary scheme 1: a) DMF, DIPEA (N,N-Diisopropylethylamine), 30°C, 5 h; b) NH$_2$OH·HCl, DIPEA in MeOH, reflux 4 h; c) Ac$_2$O, 30°C, 2 h; d) H$_2$, Pd/C, room temperature, overnight; e) NHS, EDC, 0.5 h, then Oligo-NH$_2$, 30°C, overnight

Supplementary scheme 2: a) DMSO, NEt$_3$, 30°C, overnight; b) NHS, EDC, 0.5 h, the compound 2, 30°C, overnight
Supplementary figure 1: Trypsin inhibition by a parental benzamide (2), and four bidentate conjugates of compound 73 and benzamide (8, 10, 11 and 12). The chemical structures of the inhibitors are depicted in Figure 3. The inhibition assays were performed as described in the Experimental Section.

Complete References
