Potent and Selective Kunitz Domain Inhibitors of Plasma Kallikrein Designed by Phage Display*

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Phage displaying APP1 Kunitz domain libraries have been used to design potent and selective active site inhibitors of human plasma kallikrein, a serine protease that plays an important role in both inflammation and coagulation. Selected clones from two Kunitz domain libraries randomized at or near the binding loop (positions 11-13, 15-19, and 34) were sequenced following five rounds of selection on immobilized plasma kallikrein. Invariant preferences for Arg at position 15 and His at position 18 were found, whereas His, Ala, and Pro were highly preferred residues at positions 13, 16, 17, and 19, respectively. At position 11 Pro, Asp, and Glu were favored, while hydrophobic residues were preferred at position 34. Selected variants, purified by tryptic affinity chromatography and reverse phase high performance liquid chromatography, potently inhibited plasma kallikrein, with apparent equilibrium dissociation constants (K*) ranging from ~75 to 300 pM. From sequence and activity data, consensus mutants were constructed by site directed mutagenesis. One such mutant, KALI-DY, which differed from APP1 at 6 key residues (T11D, P13H, M17A, I18H, S19P, and F34Y), inhibited plasma kallikrein with a K* = 15 ± 14 pm, representing an increase in binding affinity of more than 10,000-fold compared to APP1. Similar to APP1, the variants also inhibited Factor Xa with high affinity, with K* values ranging from ~0.3 to 15 nM; KALI-DY inhibited Factor Xa with a K* = 8.2 ± 3.5 nM. KALI-DY did not inhibit plasmin, thrombin, Factor Xa, Factor XIa, activated protein C, or tissue factor-Factor VIIa. Consistent with the protease specificity profile, KALI-DY did not prolong the clotting time in a prothrombin time assay, but did prolong the clotting time in an activated partial thromboplastin time assay >3.5-fold at 1 μM.

Plasma kallikrein plays a central role in the contact activation and kinin generating pathways (1–3). In the surface-mediated contact activation pathway, plasma prekallikrein (Fletcher Factor), thezymogen precursor, can be activated by Factor XIIa (FXIIa). Activated plasma kallikrein can then reciprocally activate Factor XII, which can activate Factor XI, ultimately resulting in fibrin formation, as well as activate the first component of the classical complement pathway. As a member of the kallikrein-kinin system, plasma kallikrein cleaves high molecular weight kininogen to produce bradykinin, a potent vasodilator. Plasma kallikrein has fibrinolytic activities as well through activation of prourokinase and plasminogen. Plasma kallikrein also stimulates neutrophils to aggregate and degranulate, releasing their lysosomal contents. Thus, plasma kallikrein is involved in both fibrin deposition and lysis, modulation of blood pressure, complement activation and support of the inflammatory response.

Protein inhibitors play critical roles in the regulation of proteases in a wide variety of physiological processes. The major physiological inhibitor of plasma kallikrein is C1 inhibitor, a serpin that results in irreversible inhibition. C1 inhibitor is also the major physiological inhibitor of FXIIa, and the complement pathway proteases C1r and C1s. α2-Macroglobulin, another major inhibitor of kallikrein, inhibits the kinin-forming function while only partially inhibiting esterolytic activity. Other serpins including antithrombin III, protein C inhibitor, and α1-antitrypsin also inhibit kallikrein to varying extents (4, 5).

Recently, ecotin, a 142-residue protein from Escherichia coli, has been shown to potentially inhibit plasma kallikrein with a K* of ~160 pm (6); however, ecotin is not totally selective and also potently inhibits Factor XIIa, Factor Xa, and human leukocyte elastase (7). Bovine pancreatic trypsin inhibitor (BPTI, aprotinin) is a well studied member of the Kunitz domain family of serine protease inhibitors that moderately inhibits plasma kallikrein with Kп of ~30 nM (8–10). However, BPTI is a more potent inhibitor of plasmin. Aprotinin has been used in a wide variety of clinical states including acute pancreatitis, septic and hemorrhagic shock, adult respiratory distress syndrome and multiple trauma; recently it has shown promise both clinically and in models of cardiopulmonary bypass (11, 12). As a broad spectrum Kunitz type serine protease inhibitor, aprotinin can prevent activation of the clotting cascade initiated by the contact activation pathway. It can also prevent activation of neutrophils and other inflammatory responses resulting from tissue damage caused by ischemia and hypoxia. These benefits are believed to be derived from its kallikrein or plasmin inhibitory activity; however, the fact that aprotinin is neither very potent nor selective make it difficult to interpret these effects.

Recently, we utilized the 58-residue Kunitz protease inhibitor domain of the Alzheimer’s amyloid β-protein precursor (APP1), which is structurally similar to BPTI (13), as a scaffold for phage display of a large library of variants to select potent and specific active site inhibitors of tissue factor-Factor VIIa (TF-FVIIa) (14, 15). In this report we have used these libraries to select and aid in the design of potent and specific active site inhibitors of human plasma kallikrein.
EXPERIMENTAL PROCEDURES

Materials—Human Factor VIIa, Factor Xa, Factor Xla, activated protein C, and thrombin were purchased from Haematologica Technologies Inc. (Essex Junction, VT). Human plasma kallikrein and Factor XIIa were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). APPI, TF7-C, and recombinant human tissue factor subdomain 243 (TF) were obtained as described previously (14). BPTI (Trasylol®) was obtained from Boehringer Mannheim. Bovine trypsin and Triton X-100 were purchased from Sigma. Bovine serum albumin (BSA), Fraction V was obtained from Calbiochem. Human plasmin, S-2302, S-2251, and S-2366 were purchased from Kabi Vitrum (Sweden), and Spectrozyme Fxa was purchased from American Diagnostica (Greenwich, CT). Cosart free amine microtiter plates (Costar, Cambridge, MA) were used to immobilize plasma kallikrein. E. coli strain XL1-Blue was from Stratagene Inc. (La Jolla, CA). All other reagents were obtained were of the highest grade commercially available.

Library Construction—Three libraries of randomized APPI variants were constructed incorporating all 20 amino acids at four or five specific positions in the APPI sequence. Codons in Library I were randomized at positions 11, 13, 15, 17, and 19. Codons in Library II were randomized at positions 11, 12, 13, and 39, and codons in Library III were randomized at positions 16, 17, 18, and 34; in addition, only codons for Arg or Lys were allowed at position 15 in Libraries II and III. All libraries and mutants except Library I contained the mutation M52A to eliminate side products due to methionine oxidation and facilitate purification. The construction of these libraries has been described (14).

Phage Selection—To produce phagedamaged particles displaying the inhibitor library, male XL1-Blue cells containing the plasmid library were infected with M13K07 helper phage (16) at a multiplicity of infection of approximately 100 helper phage/cell. Phage particles were isolated and propagated as described previously (17).

Immmobilization of Plasma Kallikrein—Costar free amine microtiter plate wells were incubated with 10 or 1μg/ml plasma kallikrein in 100 mM sodium acetate, pH 5.0. After 2 h, 10 μl of 50 μM NaCNBr was added and plates were stored at 4 °C overnight. The wells were washed three times each with 0.2 ml of TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 1 mM CaCl₂), blocked 1 h at 4 °C with 3% powdered milk in 50 mM Tris, pH 7.5, and rinsed with TBS containing 0.05% Tween 20 prior to sorting.

Selection Conditions—Inhibitor sequences with desired properties were obtained by affinity purification of phage on immobilized plasma kallikrein followed by elution and propagation of specifically bound phage. The binding reactions contained 200 μl of binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM CaCl₂ plus 0.5% BSA and approximately 1 × 10⁹ phagemid particles from a starting APPI-phage display library (amidilin-resistant, Amp<sup>-</sup>, P1<sup>-</sup> resistant, Xa<sup>-</sup>, Fxa<sup>-</sup>, and Pl<sup>-</sup>)). The phagemid library was generated as described by Lowman and Wells; Ref. 17). After mixing reactions on a microtiter plate shaker at 25 °C for 2 h, unbound phage were removed by washing 9 times with 0.3 ml of binding buffer. Phage that specifically bound to the wells were eluted by incubation with 0.2 ml of 500 mM KCN, 10 mM Tris, pH 8.0, for 1 min. The supernatant was neutralized with 25 μl of 1× Tris, pH 8.0, and an aliquot was titrated to monitor enrichment during the selection process by comparison of the number of Amp<sup>-</sup> (APPI-library phage) and Cmp<sup>-</sup> (control phage) colony-forming units. In addition, half of the eluted phage plus M13K07 were infected into XL1-Blue cells and propagated for use in subsequent rounds of phage sorting (17). Following five rounds of binding selection, clones were isolated from each library and sequenced (18).

Inhibitor Purification and Characterization—Selected variants were transformed into E. coli 27C7, a non-suppressor derivative of E. coli W3110, for expression of the free, soluble inhibitor and then purified as described previously (14). Inhibitor sequences were verified for the proper mass using a Sdxp API 3 mass spectrometer equipped with an artificial electrospray source for mass analysis.

Mutagenesis and Construction of Consensus Mutants—The construction of APPI mutants was accomplished using site-directed oligonucleotide mutagenesis (19); selected clones were analyzed by diodey sequence analysis (18). Phagemids encoding the desired mutations were then transformed into E. coli strain 27C7 for expression and purified as described previously (14).

Determination of Equilibrium Dissociation Constants—

$$V/V_o = 1 - \left[\frac{[E_i] + [I_i] + X_k}{[E_i] + [I_i] + X_k + K_i}\right] - 4 \cdot \left[\frac{[E_i] - [I_i]}{2 \cdot [E_i]}\right] \quad (Eq. 1)$$

where $V/V_o$ is the fractional activity (steady-state inhibited rate divided by the uninhibited rate), $[E_i]$ is the total plasma kallikrein active site concentration, and $[I_i]$ is the total inhibitor concentration.

Specificity Assays—Assays to test the specificity of APPI, BPTI, and Kunitz domains selected as kallikrein inhibitors against other serine proteases involved in coagulation were conducted using the following format. Aliquots (30 μl) of various inhibitors diluted to 500 nm were incubated with each protease (100 μM) in the appropriate buffer. After incubation of the substrate/inhibitor mixtures at room temperature for 2 h, the appropriate substrate (20 μl) was added, and the absorbance at 405 nm was monitored. Controls lacking inhibitor and enzyme were assayed to measure the uninhibited and substrate hydrolysis rates, respectively. The enzymes and substrates were screened in 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM CaCl₂, and 0.005% Triton X-100 as follows: thrombin (6.2 nm), 0.7 mM S2366; FXa (2.5 nm), 0.7 mM Spectrozyme FXa; FXIa (1.8 nm), 0.7 mM S2366; activated protein C (7.6 nm), 0.7 mM S2366; plasmin (32 nm), 0.7 mM S2251; Factor XIIa (14 nm), 0.7 mM S2302; TF (7.7 nm)FVIIIa (14 nm), 0.7 mM S2366 and plasma kallikrein (3.5 nm), 0.7 mM S2302. The FXa assay also contained 1 mg/ml BSA. The concentration of FXa throughout this paper refers to the concentration of active sites. For this experiment, the concentrations of thrombin, TF-FVIIIa, FXa, FXIa, and plasmin are approximate and are determined based upon the manufacturers' specifications.

Coagulation Assays—Clotting times in the prothrombin time (PT) and activated partial thromboplastin time (APTT) assays were measured using an MLA Electra 800 coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N. Y.) and Dade<sup>®</sup> reagents (Baxter Health Care Corp., Miami, FL). Actin FS was the activator in the APTT assay and rabbit thromboplastin with Ca<sup>2+</sup> was used for the PT assays.

RESULTS

Selection and Design of Kallikrein Inhibitors—APPI Kunitz domain inhibitor phage Libraries I–III were utilized to identify active sites inhibitors of plasma kallikrein. Phage were selected upon binding to plasma kallikrein immobilized in microtiter wells. Each round of selection was monitored by plating dilutions of specifically bound and eluted phage on LB plates containing either ampicillin or chloramphenicol (17). The enrichment of Kunitz domain phage (Amp<sup>+</sup>) over control phage (Cmp<sup>-</sup>) increased from 0.1-fold in the initial round of selection to 220-fold by the fifth round for Library I (amino acid positions 11, 13, 15, 17, and 19) and from 0.04-fold in the initial round of selection to 1.4 × 10⁵-fold by the fifth round for Library III (amino acid positions 15, 16, 17, 18, and 34). No enrichment of Amp<sup>+</sup> phage was observed in Library II (amino acid positions 11, 12, 13, 15, and 39).

The amino acid preferences observed at various positions from random clones in Libraries I and III are shown as a histogram in Fig. 1. From the sequence analysis of Library I, Arg was always found at the P<sub>1</sub> position (residue 15) and His, Ala, and Pro were highly favored at positions 13, 17, and 19, respectively. At position 11, a preference for Pro, Asp, or Glu was observed. From the sequence analysis of Library III, Arg was again solely selected at position 15, and His was the only
were tested in the context of the consensus residues His13, Arg15, Ala16, Ala17, His18, and Pro19. These residues held constant in this library prevented binding to kallikrein and/or (9). The lack of enrichment in Library II suggests that (39). The sequences of 18 clones from Library I and 9 clones from Library III were determined after five rounds of selection on plasma kallikrein. Only amino acids that were observed are shown. Clones with identical DNA sequences (siblings) were only counted once. Only amino acids observed above 2% were considered significant.

To further investigate amino acids selected in Libraries I and III, variants were generated by site-directed mutagenesis, which combined preferred amino acids selected from both libraries while exploring the variation in the amino acids observed at positions 11 and 34 (Fig. 1). Thus, the incorporation of Pro, Asp or Glu at position 11 and Val or Tyr at position 34 were tested in the context of the consensus residues His13, Arg15, Ala16, Ala17, His18, and Pro19.

Characterization of Kallikrein Inhibitors—Selected Kunitz domain variants were expressed in E. coli and purified to homogeneity using trypsin affinity chromatography followed by reverse phase high performance liquid chromatography. Mass spectrometry was used to verify the mass of each Kunitz domain, thus confirming the protein sequence predicted by DNA sequencing. All purified inhibitors yielded the expected mass (Tables I and II). Several variants from Libraries I and III were assayed for inhibition of plasma kallikrein and had $K_i$ values ranging from $75$ to $300 \text{ pm}$ (Table I). The site-directed consensus mutants, resulting from the combination of amino acids selected in Libraries I and III, were generally more potent kallikrein inhibitors; the most potent variant, KALI-DY, had a $K_i$ of $15 \pm 14 \text{ pm}$ (Table II). The inhibition of plasma kallikrein by APPI, BPTI, KALI-10, and KALI-DY under equilibrium conditions is shown in Fig. 2A.

The inhibition of other relevant serine proteases found in human plasma was also measured to determine the relative specificity of the Kunitz domain inhibitors. Serine proteases (1–20 nm) were assayed in the presence of 100 nm inhibitor. The fraction of remaining proteolytic activity is reported in Table III. All of the selected Kunitz domains from Libraries I and III as well as the consensus mutants inhibited FXIa, whereas none of them appreciably inhibited FXIIa, FXa, thrombin, TF-FVIIa, or activated protein C (Table III). Most of the selected Kunitz inhibitors inhibited plasmin only slightly, and moderate (>60%) inhibition was observed for KALI-38, KALI-42, and KALI-48; however, the consensus mutants did not appreciably inhibit plasmin. The degree to which FXIa was inhibited by selected inhibitors from Libraries I and III, and the consensus mutants was further investigated by measuring the $K_i$. The inhibition of FXIa by APPI, BPTI, KALI-10 and KALI-DY is shown in Fig. 2B, and $K_i$ values are reported in Tables I and II. In general, the consensus mutants are weaker inhibitors of FXIa than those selected from Libraries I and III.

KALI-DY prolonged the clotting time of the surface-mediated contact activation pathway in a concentration-dependent manner as measured by the APTT. A greater than 3.5-fold prolongation of the clotting time at 1 $\mu$m was observed with KALI-DY compared with a 2.8- and 1.8-fold prolongation observed with APPI and BPTI, respectively (Fig. 3A). In contrast, neither KALI-DY, BPTI, nor APPI appreciably prolonged the clotting time in a tissue factor-initiated PT assay (Fig. 3B).

**DISCUSSION**

Kunitz Domain Libraries—The Kunitz domain inhibitor libraries used in this report, which were randomized at or near the binding loop (positions 11–13, 15–19, 34, and 39), were previously used to identify potent inhibitors of TF-FVIIa (14, 15). The identification of potent and selective plasma kallikrein inhibitors from these same libraries demonstrates their general utility toward the selection of inhibitors for a variety of serine proteases. Although clones from Library II (positions 11, 12, 13, 15, and 39) led to random sequences, indicating a lack of enrichment, overlapping information was provided by Libraries I and III in all positions except 12 and 39. Gly is almost always found at position 12 among Kunitz domains (9) and would most likely be preferred for the inhibitors selected in this study. A new library incorporating at least some of the consensus residues from Libraries I and III could be created to investigate the amino acid preferences at position 39. Two cysteines at positions 14 and 38 form a disulfide bond found in all Kunitz domains and therefore were not changed in any of the libraries (9). The lack of enrichment in Library II suggests that (a) the residues held constant in this library prevented binding to kallikrein and/or (b) little binding energy could be recruited from this region of the Kunitz domain inhibitors.

Sequences and Affinities of Kallikrein Inhibitors—Kunitz domain inhibitors selected from Libraries I and III had apparent equilibrium dissociation constants in the range of $75$ to $300 \text{ pm}$ (Table I) and suggested a consensus sequence consisting of Pro, Asp, or Glu at position 11, His at position 13, Arg at position 15, Ala at positions 16 and 17, His at position 18, Pro...
Phage Selection of Plasma Kallikrein Inhibitors

Table I
Sequences, mass, and apparent equilibrium dissociation constants of Kunitz domains selected from randomized phage libraries with plasma kallikrein and Factor XIa

| Inhibitor | Amino acid positiona | Mass (amu) | Plasma Kallikrein Ki,b | Factor XIa Ki,b |
|-----------|----------------------|-----------|------------------------|----------------|
|           | 11 12 13 14 15 16 17 18 19 34 |          |                        |                |
|           | Calculated | Observed |                        |                |
| KALI-8c  | L g H c R a A i P f | 6411     | 6412                   | 0.074 ± 0.029  |
| KALI-10 | D g P c R a A i P f | 6373     | 6371                   | 0.122 ± 0.051  |
| KALI-13c | S g H c R a A i P f | 6385     | 6383                   | 0.203 ± 0.039  |
| KALI-30c | E g H c R a A i L f | 6443     | 6442                   | 0.258 ± 0.036  |
| KALI-46 | E g R c R a S i L f | 6478     | 6476                   | 0.175 ± 0.037  |
| KALI-48c | T g H c R a A i P f | 6399     | 6397                   | 0.209 ± 0.034  |
| KALI-38c | t g p c R A L H s Y | 6371     | 6369                   | 0.259 ± 0.106  |
| KALI-42c | t g p c R A H A s V | 6265     | 6263                   | 0.297 ± 0.170  |

* Uppercase letters represent positions that were randomized, and lowercase letters represent amino acids that were fixed in the 2 libraries.
* All Kₐ values reported are from at least three independent measurements.
* Only Arg and Lys were allowed at position 15 in Library III.

Table II
Sequences, mass, and apparent equilibrium dissociation constants of consensus Kunitz domains with plasma kallikrein and Factor XIa

| Inhibitor | Amino acid positiona | Mass (amu) | Plasma Kallikrein Ki,b | Factor XIa Ki,b |
|-----------|----------------------|-----------|------------------------|----------------|
|           | 11 12 13 14 15 16 17 18 19 34 |          |                        |                |
|           | Calculated | Observed |                        |                |
| Consensus | P             | D G H C R A A H P V Y G |          |                |
| KALI-D   | D — — — — — — — F — | 6377     | 6377                   | 0.025 ± 0.022  |
| KALI-DV  | D — — — — — — — V — | 6329     | 6328                   | 0.043 ± 0.034  |
| KALI-DY  | D — — — — — — — Y — | 6393     | 6393                   | 0.015 ± 0.014  |
| KALI-P   | P — — — — — — — V — | 6359     | 6359                   | 0.200 ± 0.099  |
| KALI-PV  | P — — — — — — — Y — | 6311     | 6310                   | 0.166 ± 0.070  |
| KALI-PY  | P — — — — — — — V — | 6375     | 6374                   | 0.136 ± 0.081  |
| KALI-E   | E — — — — — — — F — | 6391     | 6391                   | 0.103 ± 0.054  |
| KALI-EV  | E — — — — — — — V — | 6343     | 6343                   | 0.117 ± 0.059  |
| KALI-EY  | E — — — — — — — Y — | 6407     | 6407                   | 0.98 ± 0.075   |
| APP1c    | T G P C R A M I S F G | 340      | 340 ± 0.65             | 2.7 ± 1.4      |
| TF7I-C   | P G P C R A L I L F Y | 0.681    | 0.185 ± 0.185          | 0.8 ± 0.5      |
| BPT1d    | T G P C K A R I I V R | 45       | 45 ± 4                 | >300           |

* Amino acid residues that were mutagenized are shown; "—" indicates that these residues were identical to the consensus. The corresponding sequences of APP1 and BPT1 are shown for comparison.
* All Kₐ values reported are from at least 3 independent measurements.

Phage Selection of Plasma Kallikrein Inhibitors

Sequence-directed mutants were made to investigate the variability at positions 11 and 34, while incorporating highly preferred amino acids at other positions. These generally led to more potent and selective inhibitors of plasma kallikrein. In particular, mutants containing an Asp at position 11 had apparent equilibrium dissociation constants below 50 pm (Table II). One such mutant, KALI-DY, inhibited plasma kallikrein over 10,000-fold better than APP1, and 3,000-fold more potently than BPT1 (aprotinin, Trasylol®), a widely studied plasma kallikrein inhibitor (8).

Preferred Residues at the P1 Position (Residue 15)—For trypsin-like serine proteases such as plasma kallikrein, either Arg or Lys is preferred at the P1 position due to the presence of the guanidyl moiety of the P1 Arg in the crystal structure of the APP1-trypsin complex and the Arg15 BPT1-trypsinogen-Val-Val complex (24) forms hydrogen bonds directly with Asp189 and Ser190. In the crystal structure of BPT1-trypsin complex (25), the terminal amino group of the P1 Lys residue of BPT1 forms the same hydrogen bond with Ser190, however, a water molecule is used to bridge between Asp189 and the shorter Lys residue. In trypsin, Asp189 and Ser190 are likely to play important roles in ordering this water molecule to accommodate Lys at the P1 position. By contrast, plasma kallikrein has an Ala at residue 190 and is unable to aid in positioning this water molecule, thus contributing to a less favorable interaction when Lys is present at the P1 position.

The preference for Arg as the P1 residue is further supported by data for several Kunitz domain variants differing only by having either Arg or Lys here in BPT1, replacement of the Lys residue at position 19 in TF7I resulted in a 20-fold increase in affinity for plasma kallikrein (26). Furthermore, we had measured the binding affinities for APP1 variants that, in addition to inhibiting TF-FVIIa, also inhibited plasma kallikrein (15); a
comparison of two of these variants (IV-54C and IV-36B) showed that kallikrein was inhibited 95-fold more potently when Arg was at the P$_1$ position instead of Lys.

Preferred Residues on the Binding Loop—The interface between the extended binding loop of Kunitz domains and proteases is both intimate and complex, involving a diverse set of backbone and side chain interactions (10). A detailed rationale for the selection of preferred residues at randomized positions other than the P$_1$ position is speculative in the absence of a structure of plasma kallikrein. Based on comparing structure-based serine protease sequence alignments (27), we suggest the following interactions for some of the more preferred residues selected by plasma kallikrein.

There was a striking preference for His at the P$_3^\prime$ position (residue 18) among all randomized positions (Fig. 1). Residues in plasma kallikrein that are proximal to this His probably include Arg$^{39}$ and Asp$^{60}$, which could provide a favorable hydrogen bonding network and binding pocket. In comparison, the presence of Ala$^{39}$ and Phe$^{60}$ in FVIIa resulted in the selection of Ile at the P$_3$ position, whereas in human leukocyte elastase, Phe was selected to fit in the area flanked by Gly$^{39}$ and Ala$^{60}$ (14, 15, 28).

At the P$_2^\prime$ position (residue 17), the preference for Ala at position 17 is consistent with a relatively small hydrophobic pocket, which likely involves Ile$^{53}$ in plasma kallikrein. Support for Ala as a preferred residue at position 17 for plasma kallikrein also comes from variants of aprotinin, where changing Arg to Ala here resulted in a 10–15-fold improvement in affinity (29). It is unclear why Pro was preferred at the P$_4^\prime$ position (residue 19); we previously suggested that a Lys at this position would be disfavored because of charge repulsion with Arg$^{39}$ in plasma kallikrein (15). The observation of Ala as the preferred residue at the P$_4^\prime$ position (residue 16) was not unexpected since either Ala or Gly are usually found here in Kunitz domains (9).

At the P$_5$ position (residue 11), which is right at the edge of the protease/inhibitor interface, the preferred residues were Pro, Asp, and Glu (Fig. 1). However, the affinity for kallikrein was highest when Asp was present (Table II). It is tempting to propose a salt bridge here with Lys$^{129}$ in plasma kallikrein. Support for Pro as a preferred residue at position 11 for plasma kallikrein comes from variants of aprotinin, where changing Arg to Ala here resulted in a 10–15-fold improvement in affinity (29).

Specificity—When assayed for specificity toward other hu-

![Figure 2. Determination of the apparent equilibrium dissociation constants of selected Kunitz domains with plasma kallikrein and FXIa. The inhibitory activity is expressed as the fractional activity (inhibited rate/uninhibited rate) at varying inhibitor concentrations. The apparent equilibrium dissociation constants were determined by nonlinear regression analysis of the data to Equation 1. Shown in A is the fractional activity of 0.5 nM plasma kallikrein remaining in the presence of: APPI (●), BPTI (■), KALI-10 (○), and KALI-DY (▲). Shown in B is the fractional activity of 3.5 nM FXIa remaining in the presence of: APPI (●), BPTI (■), KALI-10 (○), and KALI-DY (▲). $K_i$ values are reported in Tables I and II.](image)

### Table III

| Protease $^a$ | Plasma Kallikrein | Factor Xla | Factor Xlla | Factor Xa | Thrombin | TIIa Factor VIIa | Plasmin | Activated Protein C |
|--------------|------------------|------------|-------------|-----------|-----------|----------------|---------|-------------------|
| BPT1         | 0.34             | 0.67       | 1.01        | 0.99      | 0.97      | 1.01           | 0.00    | 0.84             |
| APP1         | 0.81             | 0.03       | 0.97        | 0.89      | 0.92      | 0.60           | 0.62    | 0.87             |
| TF 7I-C      | 0.00             | 0.00       | 1.01        | 0.59      | 0.86      | 0.00           | 0.51    | 0.57             |
| KALI-8       | 0.00             | 0.00       | 1.00        | 1.13      | 0.99      | 0.94           | 0.83    | 0.89             |
| KALI-10      | 0.00             | 0.00       | 0.99        | 1.17      | 0.97      | 0.88           | 0.85    | 0.72             |
| KALI-13      | 0.00             | 0.01       | 1.00        | 0.97      | 0.96      | 0.96           | 0.65    | 0.90             |
| KALI-30      | 0.00             | 0.00       | 0.92        | 0.68      | 1.00      | 1.02           | 0.53    | 0.81             |
| KALI-38      | 0.00             | 0.02       | 0.97        | 0.52      | 1.00      | 0.45           | 0.19    | 0.97             |
| KALI-42      | 0.00             | 0.04       | 0.97        | 0.51      | 0.99      | 0.97           | 0.04    | 0.71             |
| KALI-46      | 0.00             | 0.08       | 0.97        | 1.02      | 0.99      | 0.86           | 0.64    | 0.83             |
| KALI-48      | 0.00             | 0.01       | 0.99        | 0.55      | 0.99      | 0.95           | 0.23    | 0.92             |
| KALI-D       | 0.00             | 0.02       | 1.01        | 1.17      | 0.88      | 0.91           | 1.04    | 0.68             |
| KALI-DV      | 0.00             | 0.00       | 1.02        | 1.07      | 0.89      | 0.79           | 1.06    | 0.85             |
| KALI-DY      | 0.00             | 0.03       | 1.01        | 0.87      | 0.86      | 0.86           | 1.02    | 0.99             |
| KALI-P       | 0.00             | 0.12       | 1.02        | 0.99      | 0.85      | 0.93           | 1.05    | 0.52             |
| KALI-PV      | 0.00             | 0.07       | 1.04        | 1.15      | 0.92      | 0.83           | 0.74    | 0.80             |
| KALI-PY      | 0.00             | 0.13       | 1.02        | 0.91      | 0.98      | 0.94           | 1.06    | 0.91             |
| KALI-E       | 0.00             | 0.03       | 1.00        | 0.86      | 0.95      | 0.96           | 1.10    | 0.85             |
| KALI-EV      | 0.00             | 0.01       | 0.99        | 0.77      | 0.96      | 0.97           | 1.03    | 1.01             |
| KALI-EY      | 0.00             | 0.02       | 0.99        | 0.77      | 1.00      | 1.01           | 1.07    | 1.01             |

$^a$ Each protease was assayed in the presence of 100 nM inhibitor as described under “Experimental Procedures.” The fractional activity remaining compared to an uninhibited control is reported. Boldface has been used to indicate proteases that are significantly (>60%) inhibited by the corresponding inhibitors.
Phage Selection of Plasma Kallikrein Inhibitors

The concentration of APPI ( ● ), BPTI ( ■ ), and KALI-DY ( □ ) are plotted versus the fold-prolongation of clotting time upon initiation by ellagic acid in the APTT assay (A) or by TF membranes in the PT assay (B). The uninhibited clotting times were 33.6 and 14 s for the APTT and PT, respectively.

Proteinase inhibitors from Libraries I and III also potently inhibited FXIa and to a lesser extent, plasmin (Table III). The APPI Kunitz domain itself is a potent inhibitor of FXIa, with K_i values reported from ~0.5 to 2.5 nM (30, 31). Therefore, while we significantly increased the binding affinity toward plasma kallikrein by the phage selection strategy, we did not substantially alter the inherent high affinity for FXIa. This is perhaps due to the fact that the protease domains of plasma kallikrein and Factor XIa are extremely similar, having a sequence identity of 81% (32). In particular, some of the putative binding residues mentioned above (Arg^{62}, Ile^{63}, Lys^{127}, and Lys^{320}) are conserved. Previously, we had observed that TF VIIa, a consensus TF-FVIIa inhibitor (14, 15), also inhibited plasma kallikrein, FXIa, and plasmin. In addition, the mutant human serpin α2-antitrypsin-Pittsburgh, which has an Arg at the P_1 position, potently inhibited both plasma kallikrein and FXIa (33), again suggesting that the active sites of these proteases may be similar.

The specific inhibition of plasma kallikrein improved somewhat for the consensus mutants, where inhibitor activity was completely lost toward plasmin and reduced for FXIa (Tables I–III). For example, KALI-DY inhibited plasma kallikrein over 500-fold more potently than FXIa. In addition, neither the selected inhibitors nor the consensus mutants significantly inhibited thrombin, FXa, FXIIa, activated protein C, or TF-FVIIa (Table III).

Coagulation—As predicted from the specificity assays, KALI-DY prolonged the clotting time in the APTT, a measure of the intrinsic coagulation pathway, but not the PT, a measure of the extrinsic coagulation pathway (Fig. 3). KALI-DY was significantly more effective than either APPI, a FXIa inhibitor (K_i = 2.7 nM) or BPTI, a kallikrein inhibitor (K_i = 45 nM). In addition, although plasma prekallikrein is present at 600 nM in plasma, 250 nM KALI-DY can prolong clotting by 2-fold.

Clinical Relevance—It has been suggested that the contact activation system plays a significant role in a variety of clinical states including septic shock, cardiopulmonary bypass surgery, adult respiratory distress syndrome, and hereditary angioedema (34, 35). Inhibitors of the contact system may therefore play important roles in the regulation of inflammatory and/or thrombotic disorders.

Aprotinin inhibits the contact, neutrophil, and platelet activation systems during simulated extracorporeal perfusion, as evidenced by a reduction in blood loss and kallikrein-C1-inhibitor and C1-C1-inhibitor complexes, as well as prevention of neutrophil degranulation, platelet activation and aggregation (11, 12). It has been used during lipopolysaccharide-induced endotoxic shock in pigs and prevented arterial hypotension (36). In patients with hepatic cirrhosis, aprotinin has resulted in improved renal function and filtration (37). The plasma kallikrein Kunitz domain inhibitors described here may prove useful in these and related indications.

In summary, we have developed very potent and selective inhibitors of human plasma kallikrein. Because plasma kallikrein is involved in multiple biological pathways, these selective inhibitors may allow exploration into the relative importance of this protease in its many roles.

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