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Enhancement of fibrinolysis by inhibiting enzymatic cleavage of precursor α_2-antiplasmin

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Summary

Background and objective—Resistance of thrombi to plasmin digestion depends primarily on the amount of α_2-antiplasmin (α_2AP) incorporated within fibrin. Circulating prolyl-specific serine proteinase, antiplasmin-cleaving enzyme (APCE), a homologue of fibroblast activation protein (FAP), cleaves precursor Met-α_2AP between-Pro12-Asn13- to yield Asn-α_2AP, which is crosslinked to fibrin approximately 13× more rapidly than Met-α_2AP and confers resistance to plasmin. We reasoned that an APCE inhibitor might decrease conversion of Met-α_2AP to Asn-α_2AP and thereby enhance endogenous fibrinolysis.

Methods and results—We designed and synthesized several APCE inhibitors and assessed each vs. plasma dipeptidyl peptidase IV (DPPIV) and prolyl oligopeptidase (POP), which have amino acid sequence similarity with APCE. Acetyl-Arg-(8-amino-3,6-dioxaoctanoic acid)-D-Ala-L-boroPro selectively inhibited APCE vs. DPPIV, with an apparent K_i of 5.7 nM vs. 6.1 μM, indicating that an approximately 1000-fold greater inhibitor concentration is required for DPPIV than for APCE. An apparent K_i of 7.4 nM was found for POP inhibition, which is similar to 5.7 nM for APCE; however, the potential problem of overlapping FAP/APCE and POP inhibition was negated by our finding that normal human plasma lacks POP activity. The inhibitor construct caused a dose-dependent decrease of APCE-mediated Met-α_2AP cleavage, which ultimately shortened plasminogen activator-induced plasma clot lysis times. Incubation of the inhibitor with human plasma for 22 h did not lessen its APCE inhibitory activity, with its IC_{50} value in plasma remaining comparable to that in phosphate buffer.

Conclusion—These data establish that inhibition of APCE might represent a therapeutic approach for enhancing thrombolytic activity.

Keywords

antiplasmin-cleaving enzyme inhibitor; fibrinolysis; fibroblast activation protein; prolyl oligopeptidase; Z-Proprolinal-insensitive peptidase; α_2-antiplasmin

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Introduction

Human α_2-antiplasmin (α_2AP) circulates in two N-terminal forms: precursor Met-α_2AP with N-terminal methionine and as derivative Asn-α_2AP with N-terminal asparagine, the latter rapidly crosslinked to fibrin by activated factor (FXIIIa) during blood clotting [1]. We discovered circulating antiplasmin-cleaving enzyme (APCE) and showed that it cleaves the N-terminal 12-residue peptide of Met-α_2AP to yield Asn-α_2AP [2]. The ratio of Met-α_2AP to Asn-α_2AP in normal human plasma is reported as approximately 30:70% [2–4]. Both forms inhibit free plasmin at identical rates, but during clot formation, the shorter, predominant Asn-α_2AP becomes crosslinked to fibrin by FXIIIa approximately 13× faster than the longer Met-α_2AP, and as a consequence, clot lysis rates are slowed in direct proportion to the ratio of Asn-α_2AP/Met-α_2AP in human plasma [2].

APCE circulates in human plasma essentially as a soluble form of fibroblast activation protein (FAP), a prolyl-specific serine proteinase that is a type II integral membrane protein. Based on our previous reports of virtually identical physicochemical properties for APCE and recombinant FAP, the two enzymes will be frequently referred to as APCE/FAP [2,5–7]. FAP is not expressed in normal adult tissues, but is expressed in human epithelial-derived cancers (e.g. lung, gastrointestinal, breast, etc.) [8]. It is unclear whether APCE is a derivative of membrane-inserted FAP, perhaps cleaved from some cellular origin by a ‘sheddase’ to yield soluble FAP, or instead, whether APCE is actually synthesized separately with precisely the same sequence as FAP, except without cytosolic and transmembrane segments.

We reasoned that it might be possible to specifically reduce the amount of α_2AP crosslinked into fibrin by diminishing conversion of Met-α_2AP to Asn-α_2AP with an APCE inhibitor and thereby enhance fibrinolysis. Ideally such an approach might allow the development of a therapeutic agent that increases and sustains Met-α_2AP/Asn-α_2AP ratios at levels that enhance fibrinolysis without significant fibrinolytic bleeding. We now report the design, synthesis and *in vitro* evaluation of several APCE inhibitors, one of which is highly selective for APCE vs. dipeptidyl peptidase IV (DPPIV), but less so against putative circulating prolyl oligopeptidase (POP). Poor selectivity against POP, however, appears less important, as we could not demonstrate POP activity in normal plasma samples. Nanomolar concentrations of the most selective inhibitor blocked APCE-catalyzed cleavage of Met-α_2AP, so that less α_2AP became crosslinked within fibrin as evidenced by shortened plasma clot lysis times.

Materials and methods

Reagents

APCE and Met-α_2AP were purified from fresh frozen human plasma from Sylvan Goldman Blood Institute, Oklahoma City, OK [5]. DPPIV and thrombin were from Sigma-Aldrich (Saint Louis, MO, USA). POP, urokinase (uPA) and α_2AP-depleted plasma were obtained from R & D Systems (Minneapolis, MN, USA), Abbott (Chicago, IL, USA) and Enzyme Research (South Bend, IN, USA). Tic-Pro-*AFC*, a prolyl-specific fluorescent substrate, was supplied by Vantia (Southampton, England) and Val-boroPro (Talabostat®) by Point

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Therapeutics (Boston, MA, USA). The substrates MEPLGRQLTSGP-*AMC*, acetyl-KLRP-*AMC* and prolyl boronic acid peptide inhibitors were synthesized in the Molecular Biology-Proteomics Facility, University of Oklahoma Health Sciences Center. *AFC* (7-amino-4-trifluoromethylcoumarin) and *AMC* (7-amido-4-methylcoumarin) were obtained from MP Biochemicals (Aurora, OH, USA). The University of Oklahoma Health Sciences Center Institutional Review Board approved collecting plasma samples from human volunteers for the studies reported here (IRB no. 12240).

Synthesis of peptide-like boroPro inhibitors

Potential APCE/FAP inhibitors were prepared in two steps. First a '*stalk*' structure consisting of *acetyl-Arg-peg*-(*selected P2 amino acids*) based on the sequence surrounding the P1-P1′ scissile bond in Met-α_2AP, where *peg* is defined as 8-amino-3,6-dioxaoctanoic acid, was synthesized by standard *N*-9-fluorenylmethoxycarbonyl chemistry utilizing 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the activator on (*selected amino acid*)-2-Cl-trityl resin. The protected peptide-like construct was cleaved from the synthesis support by treatment with 3:1:96 (v/v) trifluoroacetic acid-triisopropylsilane-dichloromethane for 27 min at room temperature. The solution was neutralized by adding 1:9 (v/v) pyridine/methanol and dried. The second step of the synthesis consisted of linking the protected peptide-like construct to L-boroPro-pinanediol by HATU activation (1.2 eq) of the construct's free α-carboxyl group (1.0 eq) in the presence of diisopropylethylamine (2.0 eq) in dimethylformamide for 5 min at 0 °C, followed by slow addition of L-boroPro-pinanediol-HCl (2.0 eq) in 1:1 (v/v) dichloromethane-dimethylformamide over 5 min at 0 °C. The mixture was warmed to room temperature and incubated overnight to complete the reaction. The protected peptide was purified by reversed-phase HPLC. Chemical protecting groups were removed from the peptide by treatment with 95:2.5:2.5 (v/v) trifluoroacetic acid-triisopropylsilane-water for 2 h. Finally, the inhibitor was purified by reversed-phase HPLC and characterized by electrospray mass spectrometry and amino acid analysis.

Enzyme assays

APCE (approximately 4 μg), DPPIV (approximately 2 μg) or POP (approximately 2 μg) was incubated in 25 mM sodium phosphate buffer, pH 7.5, containing 1.0 mM EDTA and 2% methanol in a total volume of 200 μL for 20 min at 22 °C. Using Tic-Pro-*AFC* (10–300 μM for APCE, 5–200 μM for DPPIV, and 40–800 μM for POP), fluorescence was monitored with time at excitation/emission wavelengths of 400/508 nm, using a black-sided 96-well plate in a BIO-TEK FL600 fluorescence plate reader (Winooski, VT, USA). For accurate assessment of kinetic parameters, saturating concentrations of substrate were used. For standard curves, dilutions of *AFC* (7-amino-4-trifluoromethylcoumarin) were prepared in the same assay buffer and corresponding fluorescence was measured. The substrate in five different concentrations (20, 40, 60, 80 and 160 μM) was mixed with four different concentrations of inhibitors around the preliminary apparent (app) K_i values [e.g. for APCE, *Inhibitor no. 6* concentrations were 2.5, 5, 10, and 20 nM for a $K_{i\ (app)}$ of approximately 5 nM]; the enzyme was added, and enzymatically released *AFC* fluorescence was recorded. Initial velocities were analyzed by non-linear regression (PRISM; GraphPad, LaJolla, CA, USA) [7].

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For assaying POP activity in human plasma, a POP-specific substrate, acetyl-KLRP-*AMC* (170 μM) was incubated in 185 μL of plasma and 15 μL of 25 mM sodium phosphate buffer, pH 7.5, containing 1.0 mM EDTA for 25 min at 22 °C. Fluorescence was monitored with time at excitation/emission wavelengths of 360/460 nm using a 96-well plate. The sensitivity and lower limit of detection for POP in plasma were determined by a published method [9].

Analysis of Met-α_2AP cleavage by APCE, DPPIV and POP

Met-α_2AP (12 μg) was incubated with 0.2 μg of APCE, DPPIV or POP in the absence or presence of inhibitor in 100 μL of 25 mM sodium phosphate-1.0 mM EDTA buffer, pH 7.5. After incubating for selected times at 37 °C, aliquots of the reaction mixtures were subjected to SDS-PAGE and Western blotting. Met-α_2AP was detected by immunostaining with an antibody specific for its N-terminal sequence, but non-reactive with Asn-α_2AP [2].

Measurement of plasma clot lysis

Met-α_2AP (8.4 μg), APCE (1.2 μg) and selected concentrations of each APCE inhibitor were added to 130 μL of α_2AP-depleted plasma to make 180 μL and incubated for 5 h at 22 °C; then 20 μL of a solution containing thrombin, $CaCl_2$, and urokinase (uPA) was added to give final concentrations of 1 unit mL^{-1}, 16 mM and 45 units mL^{-1}, respectively. A fibrin clot formed and fibrinolysis was simultaneously initiated. Turbidity was monitored at 405 nm on a microtiter plate reader (Vmax®; Molecular devices, Menlo Park, CA, USA) and plasma clot lysis time (PCLT) was defined as the midpoint between maximum absorbance and baseline absorbance [6]. The same method was used to determine PCLT of normal plasma to which neither Met-α_2AP nor APCE was added, but *Inhibitor no. 6* (50 μM) was added to one plasma sample and the same volume of buffer to the control.

Stability of inhibitor in human plasma

A citrated plasma pool was prepared from normal healthy donors. Inhibitor stability experiments utilized 200 μL of a reaction mixture containing 180 μL of citrated plasma, 300 nM Val-boroPro or acetyl-Arg-*peg*-D-Ala-L-boroPro (*Inhibitor no. 6*), 100 μM MEPLGRQLTSGP-*AMC* (substrate) and 0.8 μg APCE. Plasma samples were preincubated with the inhibitor for selected times at 22 °C before adding APCE and substrate. As a control, plasma without inhibitor was also incubated and analyzed with time. Fluorescence was recorded over 7 min at 22 °C using a BIO-TEK FL600 fluorescence plate reader at excitation/emission wavelengths of 360/460 nm.

Results

Characterization of Tic-Pro-AFC as substrate

We used a new substrate, Tic-Pro-*AFC*, for assessing APCE/FAP, DPPIV and POP, each belonging to the same clade of prolyl-specific serine proteinases and each with activity towards unique substrates. The typical synthetic substrate for APCE/FAP or POP is benzyloxycarbonyl (Z)-GP-*AMC* for which each enzyme exhibits a catalytic efficiency (k_{cat}/K_m) of 6.7×10^3 and 9.5×10^5 M^{-1} s^{-1}, respectively [7,10]. Z-GP-*AMC* is not cleaved by DPPIV [7]; however, the latter does cleave GP-*AMC* ($k_{cat}/K_m = 3.2 \times 10^4$ M^{-1} s^{-1}) [7]. GP-*AMC* is a poor substrate for APCE/FAP ($k_{cat}/K_m = 1.1 \times 10^3$ M^{-1} s^{-1}) [7] and is not

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cleaved by POP. Based on K_m and k_{cat} values in Table 1, Tic-Pro-*AFC* was readily cleaved by each enzyme, with k_{cat}/K_m for APCE/FAP being slightly higher than POP and slightly lower than DPPIV. Importantly, k_{cat}/K_m values were in keeping with those determined using Gly-Pro-*AMC* or Z-Gly-Pro-*AMC* as substrates; however, Tic-Pro-*AFC* proved more sensitive and gave more reproducible results. Thus, we chose Tic-Pro-*AFC* as the substrate for comparing the potency and selectivity of our inhibitors towards APCE/FAP, DPPIV, or POP.

Inhibitor screening

Using Tic-Pro-*AFC* as substrate and *Inhibitor no. 1*, acetyl-Gly-boroPro, we observed $K_{i(app)}$ values of 21 and 314 nM for APCE/FAP and DPPIV (Table 2), which are similar to those reported before [11]. The $K_{i(app)}$ of 23 nM for POP, however, differed from that published [11]. The explanation for differing $K_{i(app)}$ values may be that POP has more isomeric selectivity for L-boroPro isomer than does APCE/FAP or DPPIV when assessed in mixtures of acetyl-Gly-D-boroPro and acetyl-Gly-L-boroPro [12].We previously reported that Arg at P5, P6 or P7 distances from the –P1-P1′-scissile bond in APCE/FAP substrates markedly enhances kinetic efficiency when compared with those having Arg in positions P4 or P8 [7]. *Inhibitor no. 2* with Arg in P4 and *Inhibitor no. 3* with Arg positioned approximately between positions P6 and P7 were compared for inhibitor affinity towards APCE/FAP to determine whether our prior findings were useful for designing a boroPro-type inhibitor [7]. As expected, *Inhibitor no. 3* has higher affinity [$K_{i(app)}$ = 1.8 nM] towards APCE/FAP than *Inhibitor no. 2* [$K_{i(app)}$ = 17 nM]. Selectivity of *Inhibitor no. 3*, calculated by $K_{i(app)}$ (DPPIV or POP)/$K_{i(app)}$ (APCE/FAP) was 244 and 1.2 over DPPIV and POP, respectively, while selectivity of *Inhibitor no. 2* was 19 and 0.1 over DPPIV and POP, demonstrating that *Inhibitor no. 3* has greater selectivity for APCE/FAP than *Inhibitor no. 2* by 13-fold over DPPIV and 12-fold over POP. When compared with *Inhibitor no. 1*, selectivity of *Inhibitor no. 3* for APCE/FAP was improved over DPPIV by 16-fold, but was virtually the same for POP (Table 2).

Efforts were made to improve the selectivity of *Inhibitor no. 3* for APCE/FAP over POP. First, since substrate specificity data reported for APCE/FAP or POP show that Ser in P3 results in high specificity for APCE/FAP, while lowering it for POP [7,13], *Inhibitor no. 3* was synthesized with Ser at P3 (between *peg* and Gly) to yield *Inhibitor no. 4*. Its selectivity for APCE/FAP against DPPIV was only 1.3-fold better than the non-Ser-containing parent, and disappointingly, *Inhibitor no. 4* showed about the same selectivity as *Inhibitor no. 3* against POP. Second, substrates with L-Asp in P2 are reported to have the lowest specificity towards POP, but those having Gly in P2 are exclusively associated with tight specificity for APCE/FAP [7,13]. P2 Gly in *Inhibitor no. 3* was replaced with D-Asp to give *Inhibitor no. 5*, hoping that it behaved like L-Asp in POP to lessen specificity; however, probably as a result of protrusion of the carboxyl group of D-Asp from the enzyme active-site pocket, *Inhibitor no. 5*, showed markedly reduced affinity for APCE/FAP when compared with *Inhibitor no. 3*, which contained Gly in P2 (Table 2). Interestingly, *Inhibitor no. 5* also had poor selectivity against DPPIV and POP. We ultimately replaced P2 Gly in *Inhibitor no. 3* with D-Ala to give *Inhibitor no. 6*, primarily based on the report that D-Ala in P2 increased selectivity for APCE/FAP over DPPIV and POP [14]. The $K_{i(app)}$ of *Inhibitor no. 6* for

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APCE was reduced more than 1000-fold when compared with DPPIV, giving it the highest selectivity over DPPIV among the entire series of our inhibitors. When compared with POP, however, the $K_{i(app)}$ was reduced only 1.3-fold.

POP activity in human plasma

Inhibitor no. 6 has little or no selectivity for FAP/APCE over POP, but shows very high selectivity over DPPIV. Insofar as possible, we did not want to inhibit POP, given its postulated potential biologic activities. Although reported in human plasma, the use of non-specific substrates and a non-specific inhibitor called into question whether the assay measured activity attributable solely to POP or APCE, or perhaps to POP and APCE. Previous reports suggested that POP activity and Z-Pro-prolinal-insensitive peptidase (ZIP) activity as measured by cleavage of Z-GlyPro-*AMC* accounted for prolyl endopeptidase activity in bovine serum [15,16]. The ZIP form of prolyl endopeptidase was said to differ from POP, as Z-Pro-Pro-aldehyde-dimethyl acetal (Z-Pro-prolinal) inhibited POP, but not ZIP [16]. Subsequent reports from our laboratory and others, however, suggested that ZIP, albeit from bovine serum, is essentially a species homologue of APCE/FAP [2,5,16].Most reports of POP activity in human plasma/serum used Z-Gly-Pro-*AMC* or succinyl-Gly-Pro-*AMC*, both non-specific substrates, for its measurement [17–23]. Notably both substrates are cleaved by APCE/FAP and POP [5,17,21].Recently human plasma/serum POP activity was suggested to be approximately 40% POP and approximately 60% ZIP (or APCE/FAP) [17]. In that report, POP activity was defined as % inhibition of succinyl-Gly-Pro-*AMC* hydrolyzing activity in human plasma by 104 μM Z-Pro-prolinal, which was assumed to be a specific inhibitor for POP. However, we found that 32 μM Z-Pro-prolinal inhibits approximately 10% of APCE/FAP activity, thereby raising the question if the prolyl-specific serine proteinase activity inhibited by Z-Pro-prolinal – and thought to be POP – may in fact have been partially inhibited ZIP, that is, the APCE homologue. To clarify this, we designed and synthesized a POP-specific substrate, acetyl-KLRP-*AMC*, based on preferred amino acid determinations surrounding the scissile bond within the sequence of selected POP substrates [13]. We found that acetyl-KLRP-*AMC* was cleaved by POP with a K_m of 30 ± 3 μM and k_{cat} of 2.4 ± 0.1 s^{-1}, but not by DPPIV or APCE/FAP. We used the acetyl-KLRP-*AMC* to assess POP activity in a single human plasma sample (Fig. 1A) and also in a four-person pool (Fig. 1B).We then developed an assay (see Materials and methods) for POP activity in human plasma with a sensitivity of 5–10 ng mL^{-1} and a lower limit of detection of 6.3 ± 0.6 ng mL^{-1} and measured POP levels in 24 healthy persons, equally divided for gender, and randomly selected for age. In no instance were we able to demonstrate POP in human plasma, even after 22-h incubation (data not shown).

We also synthesized a POP-specific inhibitor, acetyl-KLR-L-boroPro, for separating the activities of POP and APCE/FAP, which were measured using the synthetic substrate, MEPLGRQLTSGP-*AMC* that contained the 12-residue N-terminal peptide of Met-α_2AP. We have previously reported that this peptide is a good substrate for APCE/FAP, but not for DPPIV [7]. In keeping with prior observations that POP cleaves peptides containing < 30 residues [24], the MEPLGRQLTSGP-*AMC* substrate was cleaved by POP with a K_m of 32 ± 3 μM and k_{cat} of 2.3 ± 0.1 s^{-1}. The acetyl-KLR-L-boroPro inhibitor was specific and potent as evidenced by total inhibition of POP at 100 nM in a dose-dependent manner, while

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exhibiting no inhibitory activity towards APCE/FAP or DPPIV even at 10 μM. Importantly, Fig. 2 shows that cleavage of the synthetic substrate MEPLGRQLTSGP-*AMC* by POP or APCE/FAP in non-POP-supplemented plasma was not inhibited by acetyl-KLR-L-boroPro inhibitor even at 10 μM, which is 100-fold higher than the concentration that completely inhibited POP in buffer. Of note, when plasma was supplemented with POP, only the supplemented POP activity was inhibited by the POP-specific inhibitor, acetyl-KLR-L-boroPro. However, 10 μM of our *Inhibitor no. 6*, which inhibits both POP and APCE/FAP, totally inhibited cleavage of MEPLGRQLTSGP-*AMC* in both POP-supplemented and non-supplemented plasma.

The results in Figs 1 and 2 support APCE/FAP as the only contributor of prolyl-specific endopeptidase activity in normal plasma. While DPPIV has been isolated from human plasma and characterized as soluble CD26/DPPIV [25], to our knowledge, POP has not been convincingly isolated from human plasma or serum and subjected to structure/function characterization.

Met-α_2AP as substrate for APCE/FAP, DPPIV or POP

Although evidence indicates that POP only cleaves proline-containing peptides < 30 residues, we remained concerned that the N-terminal sequence of Met-α_2AP, especially through its -Pro12-Asn13-scissile bond, might be hydrophilic and lack sufficient structure so that the 12-residue N-terminal peptide structure is randomly configured, or 'free-swimming', and therefore vulnerable to cleavage by POP just as it is by APCE. Consequently, we examined directly whether POP would cleave the -Pro12-Asn13-scissile bond of Met-α_2AP to yield Asn-α_2AP, using APCE and DPPIV as positive and negative controls, the latter only cleaving dipeptides from N-termini of substrate peptides or proteins. Figure 3 shows that APCE cleaves Met-α_2AP in a time-dependent manner, but neither DPPIV nor POP cleaved intact Met-α_2AP. Hence, these results are consistent with POP being an endopeptidase that allows only small peptides containing approximately 30 amino acids into its seven-bladed propeller-protected active-site [24].

Effect of inhibiting Met-α_2AP cleavage on plasma clot lysis

We used APCE *Inhibitor no. 6*, which shows the best selectivity against DPPIV and POP for inhibiting APCE hydrolysis of Met-α_2AP (Table 2 and Fig. 4). When Met-α_2AP was incubated with APCE in the absence of *Inhibitor no. 6* and then analyzed with an antibody that detected only its N-terminal peptide, Met-α_2AP showed significant cleavage as demonstrated by the less intense residual band in Fig. 4A. In contrast, with increasing concentrations of *Inhibitor no. 6*, the band intensity of Met-α_2AP increased in direct proportion (Fig. 4A). Figure 4B depicts densitometric analyzes of immunoblots from three experiments, which show that approximately 90 nM of *Inhibitor no. 6* is required for 50% inhibition of Met-α_2AP conversion to Asn-α_2AP.

It has not been established whether APCE inhibition promotes plasminogen activator-induced plasma clot lysis. Thus we examined the effect of *Inhibitor no. 6* on plasma clot lysis time (PCLT). Calculated PCLT values from uPA-induced PCLT profiles in Fig. 5A were as follows: control (no Met-α_2AP), 12.0 ± 0.7 min; 50 μM inhibitor, 23.9 ± 1.5 min; 5

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μM inhibitor, 28.0 ± 0.9 min; 0.5 μM inhibitor, 33.6 ± 1.7 min; and 0 μM inhibitor (no inhibitor); 40.0 ± 1.1 min. These data indicate that our APCE/FAP inhibitor enhances clot lysis in a dose-dependent manner and represent the first demonstration that clot lysis can be enhanced by an APCE/FAP inhibitor.

It is an open question whether the fibrinolysis enhancing activity we attribute to the APCE/FAP inhibitor occurs by inhibition of Met-α_2AP conversion to Asn-α_2AP or by some other unknown effect on the fibrinolytic system. Plasma samples containing Asn-α_2AP (670 nM) and APCE (38 nM) in the absence or presence of 50 μM APCE/FAP inhibitor showed essentially the same PCLT: 40.8 ± 1.2 min and 40.6 ± 1.7 min (Fig. 5B). These PCLT values are similar to the value (40.0 ± 1.1 min) from the sample containing Met-α_2AP and no inhibitor (Fig. 5A), thereby indicating that Met-α_2AP in the plasma sample is completely converted to Asn-α_2AP, when Met-α_2AP (670 nM) and APCE(38 nM) were incubated for 5 h as described in Materials and methods. In addition, the PCLT of 24.1 ± 1.9 min for the sample containing Met-α_2AP and 50 μM of *Inhibitor no. 6* (Fig. 5B) is essentially the same as the 23.9 ± 1.5 min value obtained for the plasma sample containing Met-α_2AP and a like inhibitor concentration used in Fig. 5A, underscoring the reproducibility of the inhibitor's effect.

Figure 6A shows the conversion of naturally occurring levels of Met-α_2AP to Asn-α_2AP by the native content of APCE in normal plasma. At the start of incubation, Met-α_2AP constituted 32% (approximately 320 nM) and Asn-α_2AP 68% (approximately 680 nM) of the total native plasma α_2AP (approximately 1 μM); the native APCE concentration is approximately 0.28 nM [26]. Over the course of 72 h (likely required because the normal plasma Met-α_2AP:APCE molar ratio is approximately 1200:1, rather than approximately 20:1 as in Fig. 5), all of the Met-α_2AP was converted to Asn-α_2AP. In Fig. 6B, the sample of normal plasma containing *Inhibitor no. 6* shows that the PCLT was reduced by 7% half-way through lysis and 17% at total lysis. Hence, we demonstrate in normal static plasma, containing a naturally occurring ratio of 32% Met-α_2AP/68% Asn-forms (i.e. 68% of Met-form had already been converted to Asn-form before plasma collection) that by inhibiting APCE and preventing conversion of the relatively small remaining amount of Met-α_2AP from being converted to Asn-α_2AP, easily detectable shortening of the PCLT occurred. Even though during incubation (72 h at 29 °C), proteins involved in fibrin clotting and fibrinolysis could have become somewhat less active, plasma clot lysis times were shorter with APCE *Inhibitor no. 6* present. The sum of these results indicates that the APCE inhibitor promotes uPA-induced plasma clot lysis by blocking Met-α_2AP conversion to Asn-α_2AP, thereby diminishing the content of α_2AP within fibrin and as a consequence, accelerating fibrin digestion by plasmin.

Stability of Inhibitor no. 6 in human plasma

Val-boroPro was originally developed as a DPPIV inhibitor and subsequently evaluated as an inhibitor of membrane-inserted FAP for treating patients with metastatic colorectal cancer [27]. This agent, which lacks specificity towards APCE/FAP, DPPIV or POP, also rapidly loses inhibitory activity due to cyclization in aqueous media, pH 7.8 [28]. Despite this, however, when Val-boroPro treatment was used over several days to treat cancer

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patients, Met-α_2AP/Asn-α_2AP ratios increased significantly, suggesting that *in vivo*, the drug decreased APCE/FAP activity [27,28]. This prompted us to compare the stability of Val-boroPro and our *Inhibitor no. 6* in human plasma. As described in Fig. 7, the approximately 50 ng mL^{-1} of native APCE in human normal plasma (26) was totally inhibited by 300 nM Val-boroPro at 22 °C; remaining Val-boroPro inhibitory activity was assayed at selected time intervals by adding APCE (4 μg mL^{-1}) and the enzyme substrate, MEPLGRQLTSGP-*AMC*. As expected, Val-boroPro rapidly lost inhibitory activity towards APCE/FAP within 2 h of incubation, with only approximately 10% remaining at 5 h. The inhibitory activity of *Inhibitor no. 6*, however, was sustained and remained unchanged even at 22-h incubation (Fig. 7). These findings are explained by Val-boroPro having a primary amino group on P2 that is available for cyclizing with boronic acid groups at P1 to form a B-N bond, but the length of *Inhibitor no. 6* and its lack of a P2 primary amino group obviate this possibility.

To further assess stability in human plasma, we performed the following experiment. Using 100 μM of the MEPLGRQLTSGP-*AMC* peptide substrate, we compared nine concentrations (1–10^4 nM) of *Inhibitor no. 6* for the ability to inhibit APCE/FAP (5 μg mL^{-1}) in human plasma or phosphate buffer (pH 7.5). The results were fitted to a sigmoidal curve model and yielded IC50 values of 287 ± 27 and 225 ± 12 nM, respectively, showing that the potency of *Inhibitor no. 6* towards APCE activity in human plasma is similar to that towards purified APCE in buffer alone. Hence, the effectiveness of *Inhibitor no. 6* does not appear jeopardized by binding to plasma proteins. In short, these IC50 results and those shown in Fig. 7 imply that *Inhibitor no. 6* might be useful for inhibiting APCE in human plasma.

Discussion

Reduced endogenous fibrinolytic activity is believed to contribute to survival of micro platelet-fibrin mural thrombi, so that over time, fibrin deposition becomes favored over lysis as proliferative atherosclerotic lesions develop [29–33]. With venous injury or stasis, the same reactions that give rise to endothelial-platelet-fibrin micro-thrombi are thought to initiate thrombus formation. Within the fibrin/fibrinolysis axis, changes in the concentration or function of fibrinogen, plasminogen activators, and plasminogen activator inhibitor-1 have received the most attention for roles in thrombosis. Clearly plasminogen activators are useful for selected acute occlusive thrombotic episodes, but other therapeutic measures involving the fibrinolytic system have not emerged. The ‘endgame’ of fibrinolysis is dependent on the rate at which the principal component of a thrombus, i.e., insoluble polymeric fibrin, is successfully digested and solubilized by plasmin. The serpin α_2AP is the mainstay for checking this process, but despite an intriguing and critical role in the fibrinolytic pathway, its functions have received less attention than other components of the fibrinolytic system [34]. α_2AP regulates fibrinolysis by forming inactive complexes with plasmin; inhibiting plasminogen binding to fibrin; and becoming crosslinked to fibrin by FXIIIa to provide resistance to plasmin. Precursor Met-α_2AP is made by the liver and secreted into plasma where it is cleaved by APCE to yield Asn-α_2AP that is crosslinked to fibrin approximately 13× faster than precursor Met-α_2AP. Crosslinked fibrin formed in plasma containing a preponderance of Met-α_2AP undergoes lysis by plasmin more rapidly than when Asn-α_2AP dominates [2,6,26]. Previous reports of genetic deficiencies of α_2AP

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function indicated that heterozygotes maintained clinically effective hemostasis despite moderately enhanced lysis while homozygotes experienced recurrent episodes of significant fibrinolytic bleeding [35,36]. Based on these observations and our past findings about the functional roles of APCE and α_2AP in fibrinolysis, we postulated that an effective inhibitor of APCE might maintain higher than usual Met-α_2AP/Asn-α_2AP ratios and augment endogenous fibrinolysis, but within a range of effective hemostasis.

We recently reported that the distance of a positively-charged amino acid from the P1-P1′ bond in Met-α_2AP is critical for maximizing its binding as the only proven physiologic substrate within the catalytic pocket of APCE/FAP. This proved useful in designing peptide-like constructs for inhibiting APCE/FAP and for validating our previous findings about the benefit of a positive charge properly distanced from the scissile bond [7]. We chose to add an acetyl group to the N-terminal Arg to enhance specificity for endopeptidases, and Arg spacing from P1 was accomplished by inserting a two ethylene glycol-containing unit (*peg*), which had terminal amino and carboxyl groups. Clearly the size of the peptide-like inhibitor construct (529 Da) as well as its *peg* insert should contribute to water solubility. The *peg* spacer might also enhance *in vivo* survival, while the content and spacing of the inhibitor's three amino acids should minimize antigenicity. Effective inhibitors of some members of this enzyme clade have been developed by adding Pro-boronic acid to selected single amino acids such as Val and Glu. Both compounds have been reported to have good affinities for FAP [27,37]. Unfortunately both also inhibited DPPIV *in vivo* and were exceedingly short-lived (< 1 h) as a result of cyclization of a boro-group with the N-terminal amine; however, cyclization with loss of inhibitor activity does not occur with our *Inhibitor no. 6* even after 22 h in human plasma. Based on $K_{i(app)}$ data, acetyl-Arg-*peg*-D-Ala-L-boroPro (*Inhibitor no. 6*) had the most potential for inhibiting APCE/FAP with the greatest selectivity.

Concerns remained, however, that *Inhibitor no. 6* was also effective against POP. Disappointingly, several efforts to enhance selectivity for APCE/FAP over POP by varying the length or amino acid content of the peptide-like construct did not succeed. Several reports have described structural and enzymatic properties of human POP, but its biologic functions continue uncertain [24,38,39]. Although it is said to be intracellular in a number of tissues, POP has also been reported as membrane attached [40]. In addition, POP has been noted in various body fluids, including blood, prompting the suggestion that it is released from certain cells [41–43].Most reports of POP in plasma were done before discovery of APCE.

Results from other studies that used Z-Gly-Pro-*AMC* or succinyl-Gly-Pro-*AMC*, both non-specific substrates, for measuring prolyl endopeptidase activity in human plasma/serum are not consistent with such activity being solely attributable to POP, as it has now been shown that APCE, another prolyl endopeptidase, also circulates [2,5,15,16].Moreover, given that Z-Pro-prolinal inhibits APCE in addition to POP suggests that the reported inhibition of prolyl endopeptidase activity actually represented partially inhibited APCE. Finally our inability to demonstrate POP activity in 24 different human plasma samples using a highly POP-specific substrate, led us to conclude that POP does not circulate in human plasma. We propose that if POP is confined to cytosol, the hydrophilic properties of *Inhibitor no. 6* should prevent its

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crossing hydrophobic lipid-bilayer cell membranes. Hence, our concerns about *Inhibitor no. 6* inhibiting POP in addition to APCE *in vivo* appear unwarranted.

The results in this report are the first to offer proof of a concept that a high affinity inhibitor with acceptable selectivity, particularly with respect to DPPIV, decreases APCE's proteolytic conversion of Met-α_2AP to Asn-α_2AP in a dose-response manner, with the resultant increased Met-α_2AP/Asn-α_2AP ratios corresponding to shortened lysis times for fibrin made from human plasma. As shown in Fig. 6, we have demonstrated that APCE inhibition (arrest of Met-α_2AP conversion) results in increased lysis. While these changes may seem small, persistent exposure to APCE inhibitor in the presence of normal *in vivo* protein turnover should ultimately shift total α_2AP to Met-α_2AP, which if maintained at maximal levels, would become crosslinked into fibrin much more slowly than derivative Asn-α_2AP. It is notable that *Inhibitor no. 6* has excellent aqueous solubility without attendant auto-cyclization and loss of activity as occurs with selected single amino acid-boroPro inhibitors of APCE/FAP. While other small molecule inhibitors of recombinant FAP have been reported [44], aqueous solubilities were not described despite structures suggesting a high likelihood of troublesome hydrophobicity. Clearly the overall properties of *Inhibitor no. 6* with respect to its size (529 Da), aqueous solubility, predicted low antigencity, nanomolar affinity for APCE and essentially full retention of inhibitory activity towards APCE after prolonged exposure to aqueous media suggest subsequent studies to determine if these properties are sustained *in vivo*. Just as naturally occurs in persons who are heterozygous for functionally impaired α_2AP [35,36], we conclude that it may be possible to mimic a similar long-term state of increased fibrinolysis without significant risk of bleeding and thus present potential therapeutic benefit to persons at high risk for chronic progressive intravascular fibrin deposition.

Acknowledgments

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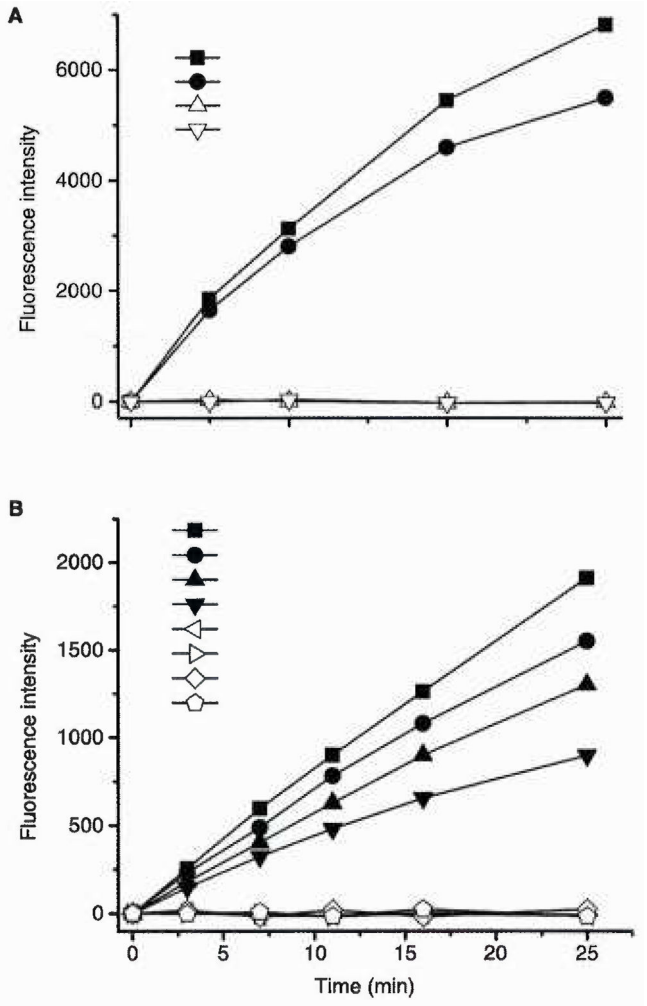

Fig. 1.

Analysis of prolyl oligopeptidase (POP) activity in human plasma. A POP-specific substrate (S), acetyl-KLRP-*AMC* (51 μM) was incubated with 0.1 μg POP – supplemented or non-supplemented normal human plasma for selected times. (A) Comparison of POP activity in a single human plasma sample vs. phosphate buffer only. (B) Substrate (S) concentration dependency of POP activity in a four-person pooled plasma sample.

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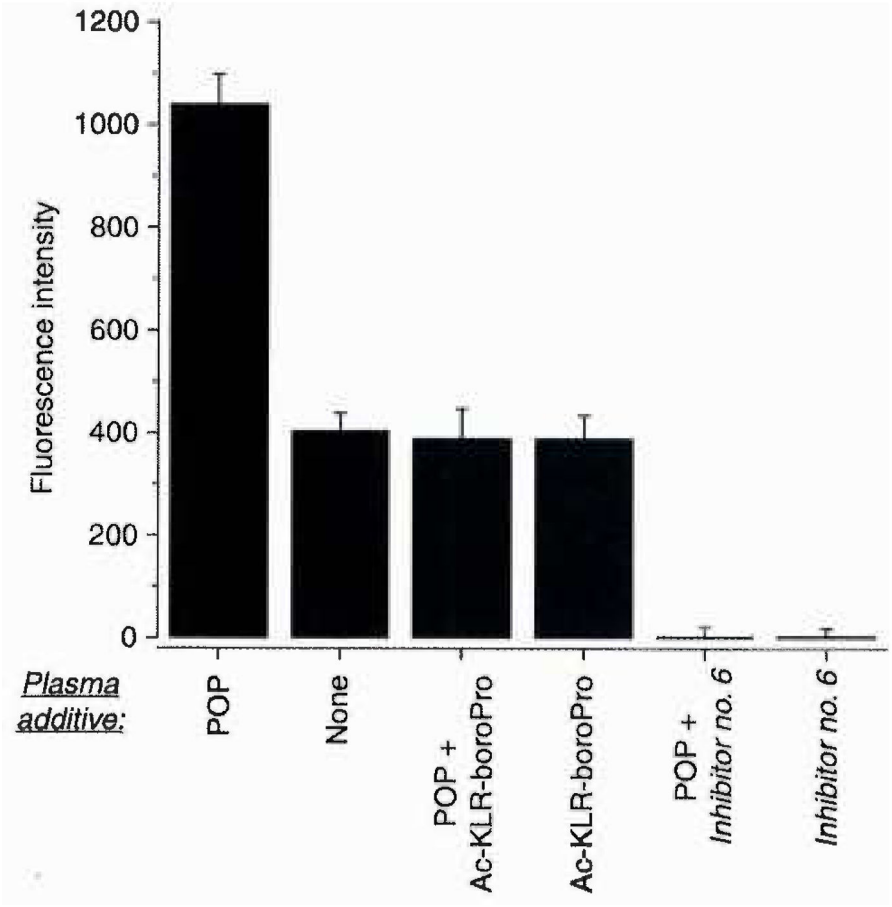

Fig. 2.

Analysis of prolyl oligopeptidase (POP) activity in human plasma using a POP-specific inhibitor. Pooled plasma from four donors was assayed in the presence and absence of supplemental POP (0.05 μg); a POP-specific inhibitor, acetyl (Ac)-KLR-L-boroPro (10 μM) or *Inhibitor no. 6* (10 μM) that inhibits both antiplasmin-cleaving enzyme (APCE) and POP. MEPLGRQLTSGP-*AMC*(100 μM) served as a substrate for APCE and POP. Each bar represents the mean ± SD of three experiments.

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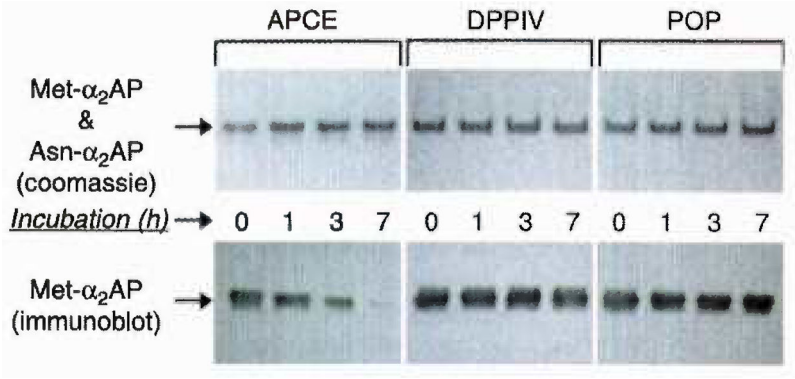

Fig. 3.

Incubation of Met-α_2AP with antiplasmin-cleaving enzyme (APCE), dipeptidyl peptidase IV (DPPIV) or prolyl oligopeptidase (POP). Each enzyme was incubated with Met-α_2AP and analyzed by SDS-PAGE and immunoblot. Top panel: Coomassie-stained reduced SDS-PAGE analyses. Bottom panel: Immunoblot with antibody specific for the N-terminal peptide of Met-α_2AP and non-reactive with Asn-α_2AP. Only APCE cleaves Met-α_2AP.

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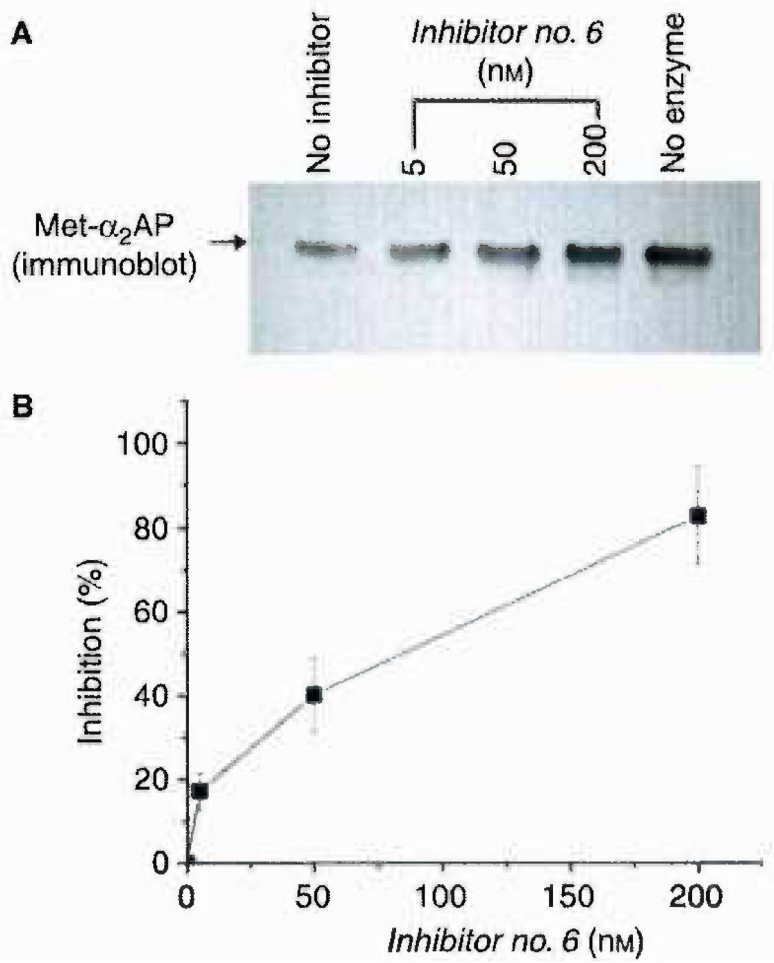

Fig. 4.

Inhibition of antiplasmin-cleaving enzyme (APCE)-catalyzed Met-α_2AP cleavage by *Inhibitor no. 6*. Met-α_2AP (12 μg) was incubated for 6 h with APCE (0.2 μg) and *Inhibitor no. 6*. Controls are indicated as either 'No Inhibitor' or 'No Enzyme'. (A) Representative immunoblot detected by antibody specific for the N-terminal peptide of Met-α_2AP and which does not react with Asn-α_2AP. (B) Densitometric analysis of immunoblot (A) for percent inhibition of Met-α_2AP cleavage by *Inhibitor no. 6*. Percent inhibition was expressed as [(densitometric value when *Inhibitor no. 6* was present – densitometric value for No Inhibitor)/(densitometric value for No Enzyme – densitometric value for No Inhibitor)] × (100). Each bar represents the mean ± SE of three experiments.

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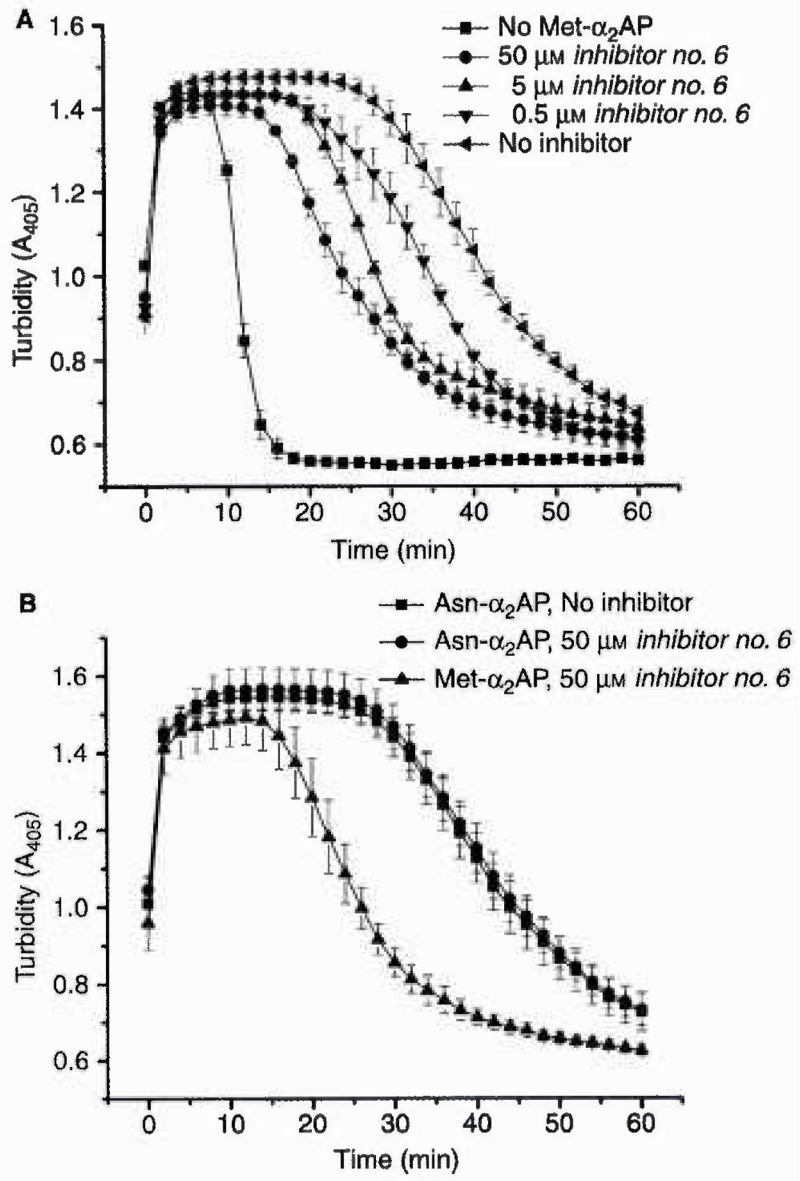

Fig. 5.

Enhancement of plasma clot lysis by *Inhibitor no. 6*. After incubating Met-α_2AP (8.4 μg) and antiplasmin-cleaving enzyme (APCE) (1.2 μg) with selected concentrations of *Inhibitor no. 6* in α_2AP-depleted plasma for 5 h, fibrin clot formation, α_2AP crosslinking to fibrin, and fibrinolysis were initiated by adding a mixture of thrombin, $CaCl_2$, and uPA. (A) Effect of *inhibitor no. 6* on clot lysis. Controls are indicated as either 'No Inhibitor' or 'No Met-α_2AP'. (B) Effect of *Inhibitor no. 6* on plasma clot lysis time (PCLT) with Asn-α_2AP (8.4 μg) and APCE (1.2 μg). As expected, the curve for 'Met-α_2AP, 50 μM *Inhibitor no. 6*' is virtually identical to that for 'Met-α_2AP, 50 μM *Inhibitor no. 6*' in (A). The data in (B) show that *Inhibitor no. 6* only affects APCE activity and none of the other fibrinolysis reactions. PCLT was defined as the midpoint between the highest and lowest absorbances in

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each clot lysis profile. Each data point for (A) and (B) is the mean ± SE of four and three experiments, respectively.

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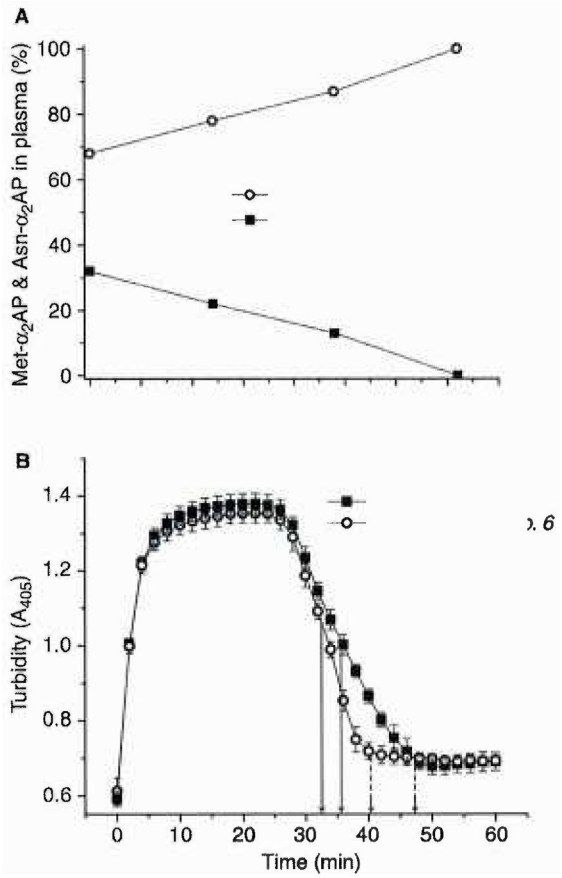

Fig. 6.

α_2AP activity in normal plasma. (A) Percent Met-α_2AP and Asn-α_2AP as determined by Edman amino terminal analyses with time during incubation of normal plasma at 29 °C. (B) Enhancement of plasma clot lysis by *Inhibitor no. 6*. Normal plasma with neither added α_2AP nor antiplasmin-cleaving enzyme (APCE) was incubated in the absence or presence of *Inhibitor no. 6* for 72 h at 29 °C after which clot formation and lysis were initiated by adding thrombin, $CaCl_2$, and uPA. Each data point is the mean ± SE of three determinations. The difference between dashed vertical lines indicates change in time for 50% lysis of inhibited vs. noninhibited control plasma while that between solid vertical lines is change in time for total lysis of inhibited vs. non-inhibited control plasma.

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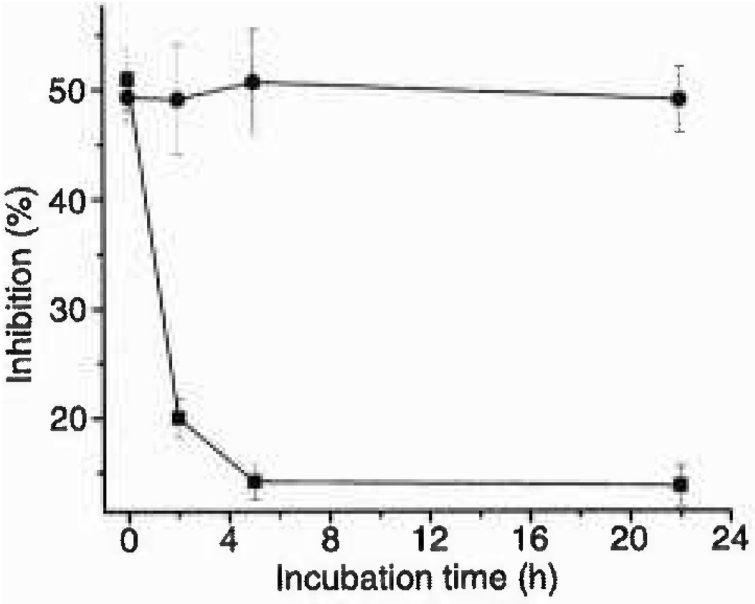

Fig. 7.

Stability of inhibitors in plasma. After incubating *Inhibitor no. 6* or Val-boroPro in plasma for selected times, antiplasmin-cleaving enzyme (APCE) inhibitory activity was assayed by adding APCE and the enzyme substrate, MEPLGRQLTSGP-*AMC*. Control plasma contained neither inhibitor. Percent inhibition was expressed as [(fluorescence for control – fluorescence with *Inhibitor no. 6*)/(fluorescence for control)] × (100).

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Table 1

Catalytic efficiencies of APCE/FAP, DPPIV and POP for Tic-Pro-*AFC*

Prolyl peptidase	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
APCE/FAP	53 ± 5	1.19 ± 0.11	2.2×10^4
DPPIV	33 ± 2	1.49 ± 0.12	4.5×10^4
POP	161 ± 12	0.76 ± 0.01	4.7×10^3

APCE, antiplasmin-cleaving enzyme; DPPIV, dipeptidyl peptidase IV; POP, prolyl oligopeptidase.

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Table 2

Inhibition constants and relative selectivity of APCE inhibitors

	APCE/FAP	DPPIV		POP	
Inhibitor*	**$K_{i(app)}$(nM)†**	**$K_{i(app)}$(nM)**	**selectivity‡**	**$K_{i(app)}$(nM)**	**selectivity**
#1 Ac-Gly-L-boroPro	20.7 ± 0.9	314 ± 13	15.2	23.3 ± 1.1	1.1
#2 Ac-Arg-Gly-Gly-L-boroPro	17.2 ± 1.5	322 ± 29	18.7	1.6 ± 0.1	0.1
#3 Ac-Arg-*peg*-Gly-L-boroPro	1.8 ± 0.2	440 ± 29	244	2.1 ± 0.2	1.2
#4 Ac-Arg-*peg*-Ser-Gly-L-boroPro	2.7 ± 0.3	861 ± 66	319	2.9 ± 0.3	1.1
#5 Ac-Arg-*peg*-D-Asp-L-boroPro	1377 ± 116	7129 ± 221	5.2	23.9 ± 2.1	0.02
#6 Ac-Arg-*peg*-D-Ala-L-boroPro	5.7 ± 0.4	6136 ± 408	1076	7.4 ± 0.6	1.3

** Ac* represents an acetyl group; *peg* indicates a short ethyleneglycol unit, 8-amino-3,6-dioxaoctanoic acid.

† Data represent the best-fit value ± the standard error.

‡ Relative selectivity was expressed as $K_{i(app)}$ (DPPIV or POP)/$K_{i(app)}$ (APCE/FAP).

APCE, antiplasmin-cleaving enzyme; DPPIV, dipeptidyl peptidase IV; POP, prolyl oligopeptidase.