Supporting Information

Guarnieri et al. 10.1073/pnas.1001349107

SI Methods

Synthesis of Compounds. The small molecule inhibitors, the peptide, and the peptide-inhibitor constructs were synthesized by Sai Advantium Pharma. All final products were purified by preparative HPLC, and the average molecular masses were confirmed by liquid chromatography/mass spectrometry (LC/MS). Table 1 summarizes the compounds and their structures and molecular masses.

Formation of small molecule inhibitors. Small molecule inhibitors in the form of tripeptidyl difluoro-β-ketoamides (1) were synthesized by the numbered scheme in Fig. S2. As the first step, the commercial *N*-protected dipeptide Cbz-Val-Pro-OH, **1**, was dissolved in tetrahydrofuran (THF), followed by the addition of 4-methylmorpholine and the coupling agent isobutyl chloroformate. The carboxyl group was activated by the isobutyl chloroformate to form a mixed carboxylic-carbonic anhydride intermediate. The intermediate was reacted with the commercial amino alcohol L-valinol, **2**, to give the *N*-protected tripeptide alcohol, **3**. A solution of the primary alcohol, **3**, in dichloromethane (DCM) was subjected to Swern oxidation, in which oxalyl chloride and dimethyl sulfoxide were added to generate a sulfonium salt that reacted with the alcohol to form an alkoxysulfonium ion intermediate. The addition of the base *N*,*N*-diisopropylethylamine caused the decomposition of the intermediate to the aldehyde, **4**, while reducing epimerization at the carbon adjacent to the newly formed carbonyl. The aldehyde, **4**, was then mixed with ethyl bromodifluoroacetate, **5**, in a suspension of zinc/THF. The in situ formation of an ester-stabilized organozinc compound (Reformatsky reagent) led to nucleophilic addition to the aldehyde group, transforming it into an α,α-difluoro-β-hydroxy ester group. After purification by preparative HPLC, the ester, **6**, was reacted with an ethanol solution of benzylamine, **7**, and was converted by nucleophilic acyl substitution to the ketoamide, **8**. The resulting ketoamide, **8**, was the parent compound for the subsequent synthesis of the three small molecule inhibitors (X0, X1, and X2) of this study.

The formation of the small molecule inhibitor X0 required one additional step. The hydroxyl group on the β-carbon of the ketoamide, **8**, was oxidized to a carbonyl group using Dess–Martin periodinane [1,1,1-Tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1*H*)-one] in a DCM solution with trifluoroacetic acid (TFA) as an accelerant. The purified product, **9**, was the small molecule inhibitor X0 that incorporated the *N*-protective benzyloxycarbonyl (Cbz) group (Table 1). HPLC analysis showed a mixture of epimers that could not be separated (Fig. S3*A*). Epimerization was also detected by proton NMR (^{1}H-NMR) spectroscopy [e.g., δ 1.61 (23 H), R-CH_3; δ 7.32 (19 H), R-C_6H_5]. Examination of the total ion current (TIC) trace (Fig. S3*B*) from LC/MS analysis showed two peaks for which the full mass spectra (Fig. S3 *C* and *D*) revealed a common ion (*m*/*z* 615; $M + H^+$) supporting the predicted molecular mass of 614 Da.

The small molecule inhibitor X1 was prepared by a more extensive series of steps. The *N*-protective group on the ketoamide, **8**, was removed by hydrogenolysis with palladium hydroxide in ethyl acetate. A solution of the deprotected amine, **10**, in tri-ethylamine/DCM was mixed with a linker molecule of monoethyl oxalyl chloride, **11**. The ensuing reaction between the amino nitrogen and the carbonyl carbon of the linker resulted in the formation of the alcohol, **12**. The presence of tri-ethylamine in the solution helped to catalyze the coupling and remove HCl by-product. After purification by preparative HPLC, the secondary alcohol, **12**, was oxidized to the ketone, **13**, under mild conditions using Dess–Martin periodinane/DCM with TFA. The ketone, **13**, was solubilized in methanol/water, and the ester end group was hydrolyzed with NaOH to the sodium salt, followed by acidification with HCl to the acid, **14**. The purified product, **14**, was the small molecule inhibitor X1 (Table 1). HPLC indicated some epimerization of the product, and LC/MS verified the predicted molecular mass of 552 Da (*m*/*z* 553; $M + H^+$).

Except for the choice of linker molecule, a similar series of steps was followed for the synthesis of the small molecule inhibitor X2. In this instance, an ester linker of monoethyl malonate, **15**, was combined with the deprotected amine, **10**, in THF. The carboxyl group of the linker was activated by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and a highly reactive *O*-acyl urea intermediate was formed between linker and activator. The formation of the intermediate enhanced the main reaction between the amino nitrogen and the carbonyl carbon of the linker, and the addition of 1-hydroxybenzotriazole (HOBt) and 4-methylmorpholine suppressed racemization and side reactions. The product of the main reaction was the secondary alcohol, **16**, which was subsequently oxidized to the ketone, **17**, and hydrolyzed to the acid, **18**, by the previously described steps. The purified product, **18**, was the small molecule inhibitor X2 (Table 1). As shown by HPLC, the product was a mixture of epimers, with a predicted molecular mass of 566 Da confirmed by LC/MS (*m*/*z* 567; $M + H^+$).

Formation of peptide. The peptide (SP-B1-25) was synthesized according to a sequence defined by the first 25 *N*-terminal residues of human surfactant protein B (Table 1). The chain was assembled by solid-phase peptide synthesis using Wang resin (4-benzyloxybenzyl alcohol resin) and 9-fluorenylmethyl carbamate (Fmoc) chemistry. At the end of the synthesis, the *N*-protecting Fmoc group was removed with a mild base (piperidine), and the resin-immobilized peptide (SP-B1-25-resin) was either treated with TFA to release the peptide or retained for further reaction with the small molecule inhibitors (see next section). HPLC results for the peptide product (Fig. S4*A*) showed a purity of 97%. The TIC trace (Fig. S4*B*) from LC/MS analysis of the peptide located a major peak, and full mass spectra (Fig. S4 *C* and *D*) at two locations within the peak verified the predicted molecular mass of 2,927 Da for the peptide (*m*/*z* 976–977; $M + 3H^+$).

Formation of peptide-inhibitor constructs. The formation of the peptide-inhibitor construct was initiated by mixing the deprotected SP-B1-25-resin with a small molecule inhibitor (X1 or X2) in dimethyl-formamide (DMF). The phosphonium coupling reagent [benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP)], together with a mild base (4-methylmorpholine), was added to the mixture to activate the carboxyl group of the inhibitor to an ester group. The ester species was relatively stable and less prone to racemization, and the carbonyl carbon was more vulnerable to nucleophilic attack by the amino nitrogen at the end of the deprotected peptide. The subsequent reaction produced the desired peptide-inhibitor construct (SP-B1-25-X1 or SP-B1–25-X2; Table 1), which was removed from the resin with a TFA cleavage solution. LC/MS measurements validated the predicted molecular masses of 3,461 Da for the SP-B1–25-X1 construct (*m*/*z* 1155; $M + 3H^+$) and 3,475 Da for the SP-B1-25-X2 construct (*m*/*z* 1160; $M + 3H^+$).

In Vitro Assessment of Elastase Inhibitors. Fresh stock solutions of the samples were prepared as 1- to 2-mg/mL solutions in dimethyl sulfoxide (DMSO) before each experiment. All of the compounds appeared to be soluble in DMSO on the basis of visual inspection. Additional dilutions of the stock solutions were made with buffer

containing 0.5% DMSO. Aggregation on the macroscopic scale, such as cloudiness when the DMSO solutions were diluted with buffer, was not observed as long as the final solution contained at least 0.3% DMSO.

The enzyme and substrates were obtained from Elastin Products Company and consisted of HNE (human purulent sputum; 29.5 kDa), insoluble elastin (bovine neck ligament; particle size <37 μm), and synthetic substrate *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (Suc-Ala_3-*p*NA; molecular weight 451.5). Heparin (porcine intestinal mucosa; 17–19 kDa) was supplied by Sigma-Aldrich. Dulbecco's PBS without calcium or magnesium salts was from Invitrogen, and other buffer components were from Sigma-Aldrich. All water was 0.22-μm filtered Milli-Q water.

Elastin solubilization test. HNE digestion of elastin was measured with and without inhibitor at three inhibitor/enzyme (I/E) molar ratios of 1, 5, and 12–14. Heparin was included at an I/E ratio of 2.3 because of past experience with this compound (2). Reaction mixtures (1.020 mL) in duplicate containing 0.98 mg/mL elastin, 120 nM HNE, and an appropriate amount of inhibitor in PBS with 0.5% DMSO were slowly rotated (6 rpm) at 37 °C for 4 h. Mixtures were then filtered (Millipore Ultrafree-CL; 0.22 μm), and filtrates were analyzed with the Fastin Elastin assay (Biocolor), a quantitative dye-binding method using 5,10,15,20-tetraphenyl-21,23-porphine tetrasulfonate (TPPS) to detect soluble elastin. Measurements were averaged for each condition and plotted as relative rate (v_i/v_o = digestion with inhibitor/digestion without inhibitor). A second method used ^{3}H-elastin and 75 nM HNE as previously described (3).

Concentration-response test. HNE hydrolysis of Suc-Ala_3-*p*NA was measured with and without inhibitor at three I/E molar ratios of 1, 10, and 100. SP-B1-25 was excluded from testing because it was inactive in the elastin solubilization test. The remaining five inhibitors, together with heparin, were screened in this abbreviated concentration-response test. All reaction mixtures (250 μL) were prepared in duplicate in a 96-well microplate. Each well contained 1.50 mM Suc-Ala_3-*p*NA, 120 nM HNE, and an appropriate amount of inhibitor in 0.10 M Tris/Tris-HCl, 0.093 M NaCl (pH 8.0, ionic strength 0.15 M at 27 °C) plus 0.5% DMSO. The microplate was inserted into a SPECTRAmax tunable microplate reader (Molecular Devices) at 27 °C for 1 h. Release of the hydrolytic product 4-nitroaniline was measured optically at 410 nm every 30 s. The resulting absorbance readings were averaged for each condition and plotted as a function of time. The steady-state reaction rate (v) was determined from the slope of the plot between 40 and 60 min. The resulting trend in the relative rate (v_i/v_o = rate with inhibitor/rate without inhibitor) vs. the I/E ratio was used to characterize the type of inhibition (linear or hyperbolic; tight binding or not tight binding) for each compound.

Substrate-response test. For each of the five inhibitors, HNE hydrolysis of Suc-Ala_3-*p*NA was measured with and without inhibitor at a constant I/E molar ratio and six substrate concentrations (0.6–6.0 mM). The HNE concentration was maintained at 120 nM for all conditions, and the concentration of each inhibitor was selected to give a spread of measurable reaction rates over the given substrate range. For this reason, the I/E ratio varied from a high of 10 (SP-B1-25-X1 and SP-B1-25-X2) to lower values of 2 (X1) and 0.5 (X0 and X2). After the appropriate components were added to the microplate, the same general procedure described for the concentration-response test was followed for the substrate-response test. However, once the steady-state reaction rates (v) were calculated for each system, the data were fit by nonlinear regression to the Michaelis–Menten equation. The resulting best-fit values for the apparent maximal velocity (V_{ma}) and the apparent Michaelis constant (K_{ma}) were compared with similarly computed values of V_m and K_m for the control (no inhibitor). This comparison yielded the inhibition mechanism (competitive, noncompetitive, uncompetitive, or mixed) for each inhibitor, which was visually illustrated with a Lineweaver–Burk plot. For those compounds that were found to be classical inhibitors (linear with no tight binding), the value of the dissociation constant (K_i) was determined from the initial inhibitor concentration $[I]_o$ and the ratios (K_{ma}/K_m) and (V_{ma}/V_m), depending on the mechanism. In the case of a classical, competitive inhibitor, this relation was $K_i = [I]_o/(K_{ma}/K_m - 1)$. For the remaining inhibitors that were nonclassical inhibitors (tight binding or hyperbolic), concentration-response tests with more data points, particularly at very low inhibitor concentrations, were required for a valid estimate of K_i. However, as a first approximation, the values of K_i were estimated assuming classical inhibition.

1. Veale CA, et al. (1997) Orally active trifluoromethyl ketone inhibitors of human leukocyte elastase. *J Med Chem* 40:3173–3181.
2. Spencer JL, Stone PJ, Nugent MA (2006) New insights into the inhibition of human neutrophil elastase by heparin. *Biochemistry* 45:9104–9120.
3. Stone PJ, Lucey EC, Snider GL (1990) Induction and exacerbation of emphysema in hamsters with human neutrophil elastase inactivated reversibly by a peptide boronic acid. *Am Rev Respir Dis* 141:47–52.

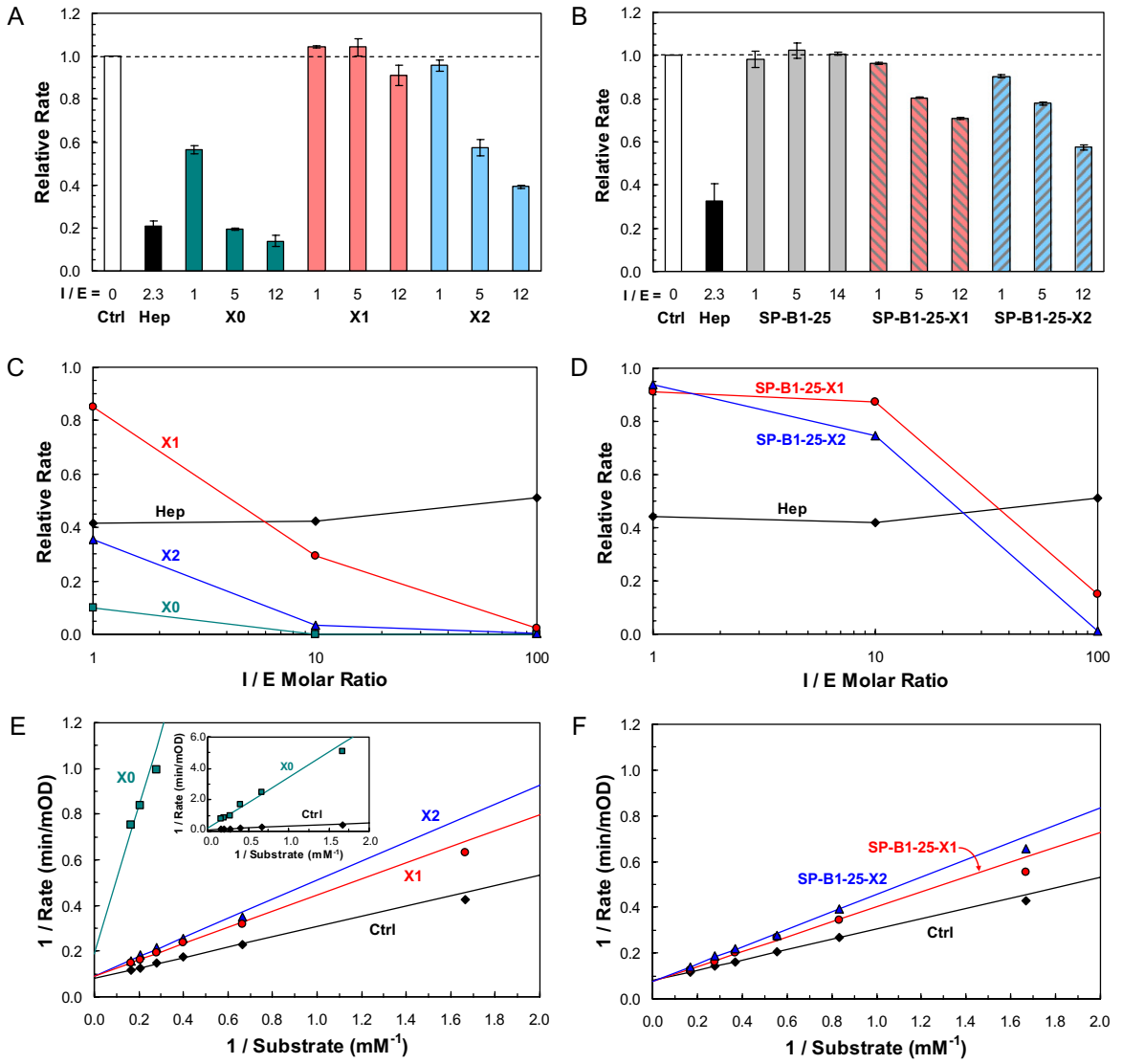

Fig. S1. In vitro assessment of HNE inhibitors. Elastin solubilization tests are shown for (*A*) the small molecule inhibitors and (*B*) the peptide-inhibitor constructs. Bar height equals the average of duplicate readings; error bar shows the propagation-of-error estimate using standard errors. Relative rate (v_i/v_o) = (digestion with inhibitor)/(digestion without inhibitor); Ctrl, control (no inhibitor); Hep, heparin; *I/E*, inhibitor/enzyme molar ratio. Reaction conditions: $[E]_o$ = 120 nM HNE; $[S]_o$ = 0.98 mg/mL elastin; buffer, Dulbecco's PBS without calcium or magnesium salts (pH 7.1, ionic strength 0.15 M) plus 0.5% DMSO; T = 37 °C; t = 4 h; V = 1.020 mL. Concentration-response tests are shown for (*C*) the small molecule inhibitors and (*D*) the peptide-inhibitor constructs. Each data point equals the average of duplicate readings. Relative rate (v_i/v_o) = (rate with inhibitor)/(rate without inhibitor); Hep, heparin; *I/E*, inhibitor/enzyme molar ratio. Reaction conditions: $[E]_o$ = 120 nM HNE; $[S]_o$ = 1.50 mM Suc-Ala_3-*p*NA; buffer, 0.10 M Tris/Tris-HCl, 0.093 M NaCl (pH 8.0, ionic strength 0.15 M at 27 °C) plus 0.5% DMSO; T = 27 °C; t = 1 h; V = 250 μL. Substrate-response tests are shown for (*E*) the small molecule inhibitors and (*F*) the peptide-inhibitor constructs. Each data point equals the average of duplicate readings. Lines were generated with kinetic parameters (K_m, V_m) determined from nonlinear regression of the data to the Michaelis–Menten equation. Reaction conditions: $[E]_o$ = 120 nM HNE; $[S]_o$ = 0.6–6.0 mM Suc-Ala_3-*p*NA; $[I]_o$ = 60 nM (X0, X2), 240 nM (X1), and 1200 nM (SP-B1-25-X1, SP-B1-25-X2); buffer, 0.10 M Tris/Tris-HCl, 0.093 M NaCl (pH 8.0, ionic strength 0.15 M at 27 °C) plus 0.5% DMSO; T = 27 °C; t = 1 h; V = 250 μL.

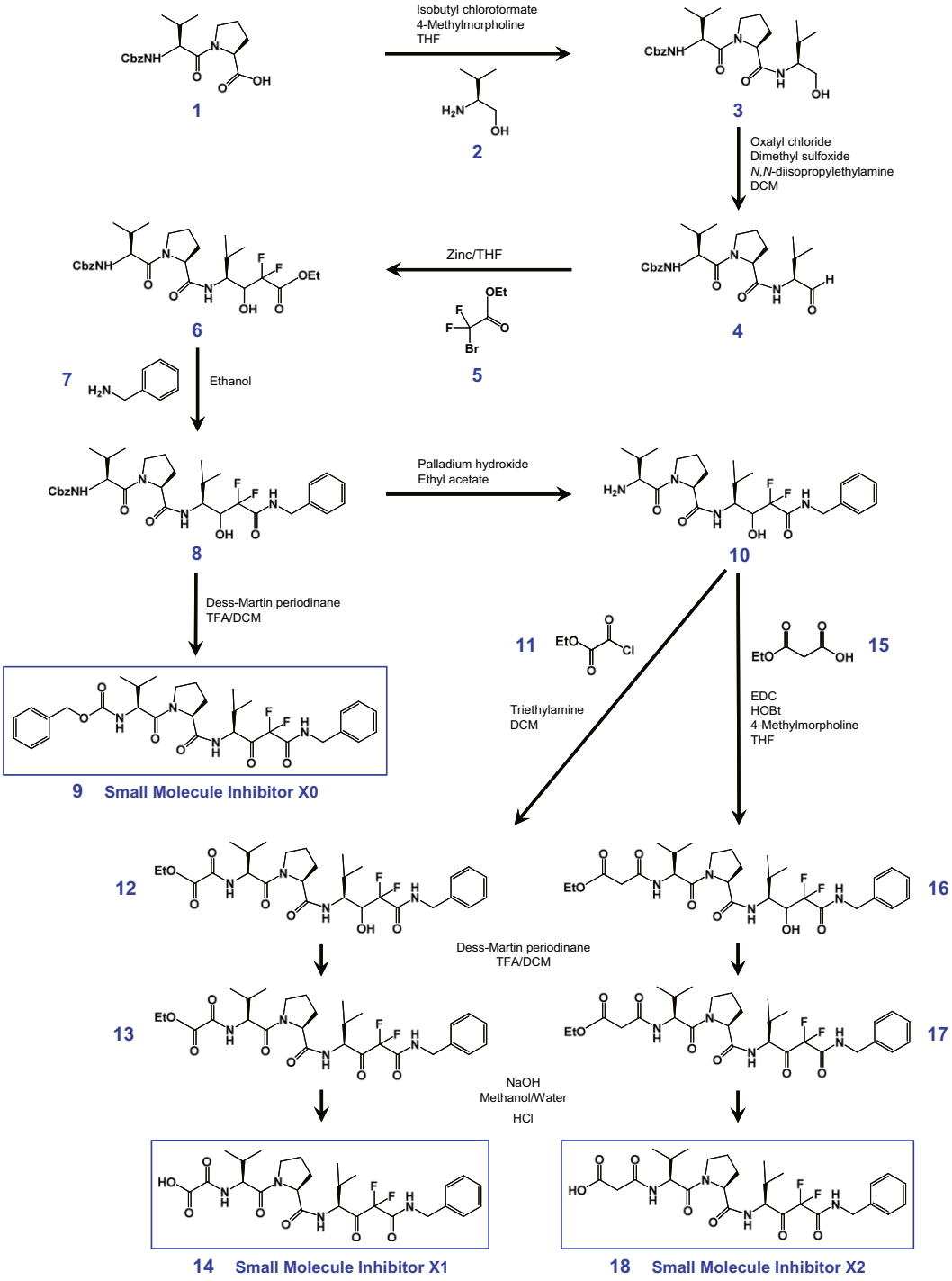

Fig. S2. Synthesis scheme for the preparation of the small molecule inhibitors X0, X1, and X2.

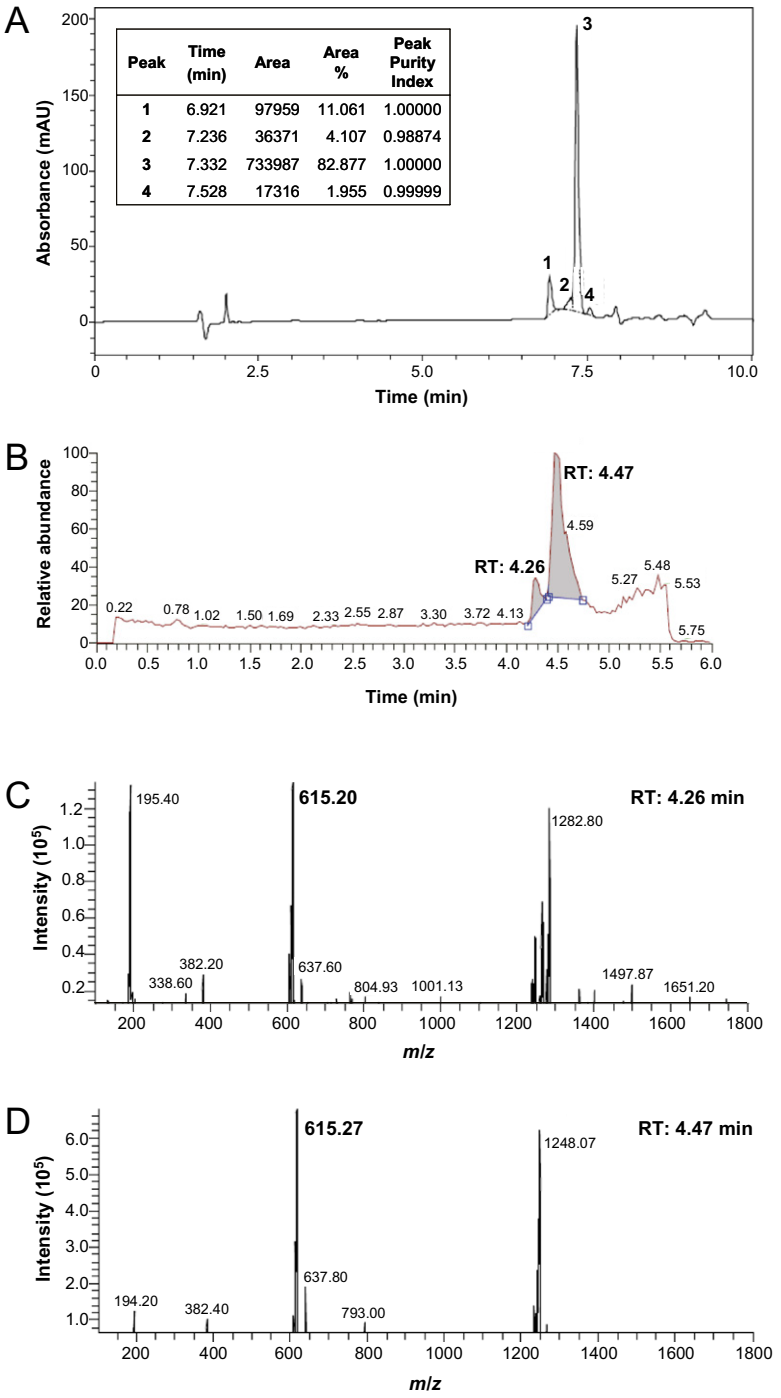

Fig. S3. HPLC and LC/MS results for the small molecule inhibitor X0. (*A*) HPLC chromatogram obtained under the following conditions: column, YMC ODS-A (fully endcapped C18 packing; YMC Co.) 150 mm × 4.6 mm × 5 μm; mobile phase, (*A*) 0.05% TFA in water and (*B*) 0.05% TFA in acetonitrile; gradient, 10% B to 80% B in 5 min, hold for 2 min, 7.01–10 min 10% B; column temperature, 30 °C; flow rate, 1.2 mL/min; injection volume, 10 μL; UV detector, 220 nm. (*B*) TIC chromatogram from LC/MS analysis. (*C*) Full mass spectrum obtained after 4.26 min in LC/MS analysis. (*D*) Full mass spectrum obtained after 4.47 min in LC/MS analysis. All mass spectra were collected using positive ion electrospray mass spectrometry. *m/z*, ion mass (Da)/ion charge; RT, retention time.

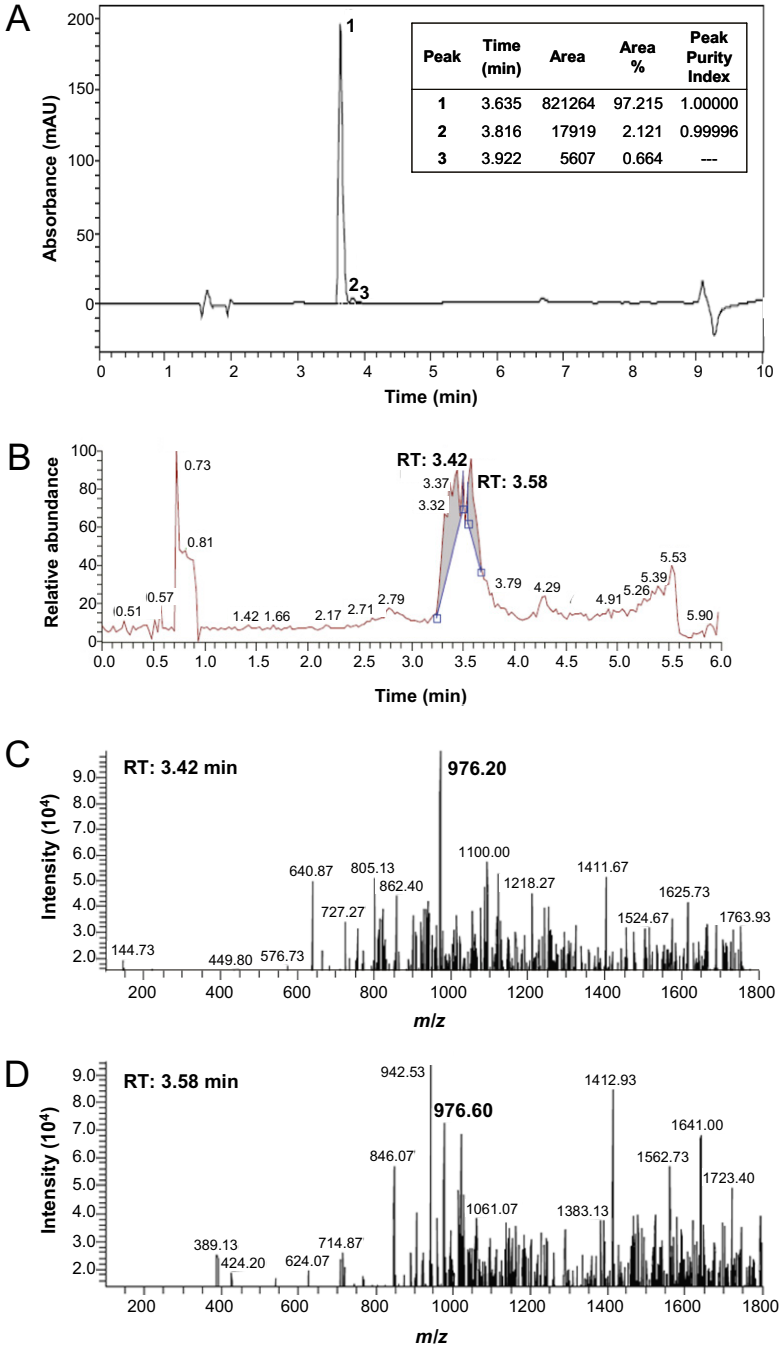

Fig. S4. HPLC and LC/MS results for the peptide SP-B1-25. (*A*) HPLC chromatogram obtained under the following conditions: column, YMC ODS-A (fully endcapped C18 packing; YMC Co.) 150 mm × 4.6 mm × 5 μm; mobile phase, *A*: 0.05% TFA in water and *B*: 0.05% TFA in acetonitrile; gradient, 10% B to 80% B in 5 min, hold for 2 min, 7.01–10 min 10% B; column temperature, 30 °C; flow rate, 1.2 mL/min; injection volume, 10 μL; UV detector, 220 nm. (*B*) TIC chromatogram from LC/MS analysis. (*C*) Full mass spectrum obtained after 3.42 min in LC/MS analysis. (*D*) Full mass spectrum obtained after 3.58 min in LC/MS analysis. All mass spectra were collected using positive ion electrospray mass spectrometry. *m/z*, ion mass (Da)/ion charge; RT, retention time.

Table S1. Kinetic parameters for in vitro inhibition of HNE by synthesized compounds

Inhibitor	$[I]_o$, nM	K_m, mM	V_m*, 10^{-8} M/s	K_{ma}/K_m	V_{ma}/V_m	K_i, nM
Control	—	2.9 ± 0.3[†]	4.1 ± 0.2[†]	—	—	—
X0	60	17 ± 10[‡]	1.7 ± 0.8[‡]	5.9	0.41	12[§]
Control	—	2.9 ± 0.3	4.0 ± 0.2	—	—	—
X1	240	4.0 ± 0.4	3.6 ± 0.2	1.4	0.89	630[§]
X2	60	4.6 ± 0.9	3.5 ± 0.4	1.6	0.88	97[§]
Control	—	2.9 ± 0.2	4.1 ± 0.1	—	—	—
SP-B1-25-X1	1,200	4.4 ± 0.9	4.3 ± 0.6	1.5	1.0	2,300
SP-B1-25-X2	1,200	5.0 ± 0.5	4.2 ± 0.2	1.7	1.0	1,600

Kinetic parameters (K_m, V_m) were determined by nonlinear regression of the data (Fig. S1 *E* and *F*) to the Michaelis–Menten equation. Reaction conditions: $[E]_o$ = 120 nM HNE; $[S]_o$ = 0.6–6.0 mM Suc-Ala_3-*p*NA; $[I]_o$, initial inhibitor concentration (listed in table); buffer, 0.10 M Tris/Tris-HCl, 0.093 M NaCl (pH 8.0, ionic strength 0.15 M at 27 °C) plus 0.5% DMSO; T = 27 °C; t = 1 h; V = 250 μL.

*Rate was multiplied by 3.205×10^{-9} to convert from (mOD/min) to (M/s).

[†]Best-fit value (±SE).

[‡]Values for inhibitor system are apparent kinetic parameters, K_{ma} and V_{ma}.

[§]Value of K_i was overestimated by assuming classical inhibition for a tight-binding inhibitor.