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A selective reversible azapeptide inhibitor of human neutrophil proteinase 3 derived from a high affinity FRET substrate

Christophe Epinette [a], Cécile Croix [b], Lucie Jaquillard [c], Sylvain Marchand-Adam [a,d], Christine Kellenberger [e], Gilles Lalmanach [a], Martine Cadene [c], Marie-Claude Viaud-Massuard [b], Francis Gauthier [a], Brice Korkmaz [a,*]

[a] INSERM U-1100 "Pathologies Respiratoires: Protéolyse et Aérosolthérapie", Université François Rabelais, Faculté de Médecine, 37032, Tours, France
[b] Génétique, Immunothérapie, Chimie et Cancer, Université François Rabelais, CNRS-UMR 6239, 37032, Tours, France
[c] Centre de Biophysique Moléculaire, UPR 4301 CNRS, 45071, Orléans, France
[d] Centre Hospitalier Régional Universitaire, service de pneumologie, Université François Rabelais, 37032, Tours, France
[e] Architecture et Fonction des Macromolécules Biologiques, CNRS-UMR 6098, 13288, Marseille, France

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ABSTRACT

The biological functions of human neutrophil proteinase 3 (PR3) remain unclear because of its close structural resemblance to neutrophil elastase and its apparent functional redundancy with the latter. Thus, all natural inhibitors of PR3 preferentially target neutrophil elastase. We have designed a selective PR3 inhibitor based on the sequence of one of its specific, sensitive FRET substrates. This azapeptide, azapro-3, inhibits free PR3 in solution, PR3 bound to neutrophil membranes, and the PR3 found in crude lung secretions from patients with chronic inflammatory pulmonary diseases. But it does not inhibit significantly neutrophil elastase or cathepsin G. Unlike most of azapeptides, this inhibitor does not form a stable acyl–enzyme complex; it is a reversible competitive inhibitor with a K_i comparable to the K_m of the parent substrate. Low concentrations (60 μM) of azapro-3 totally inhibited the PR3 secreted by triggered human neutrophils (200,000 cells/100 μL) and the PR3 in neutrophil homogenates and in lung secretions of patients with lung inflammation for hours. Azapro-3 also resisted proteolysis by all proteases contained in these samples for at least 2 h.

1. Introduction

Proteinase 3 (PR3) also called myeloblastin, is one of the three neutral serine protease (NSP) stored in the primary/azurophil granules of neutrophils, together with neutrophil elastase (HNE) and cathepsin G (CG). All three contribute to the proteolytic potential of these immune cells and are involved in lung tissue degradation during chronic inflammatory lung diseases [1,2]. However, most reports on PR3 focus on its role as a target antigen for anti-neutrophil cytoplasmic antibodies (ANCAs) during small vessel vasculitides, a panel of inflammatory diseases that are characterized by necrotizing inflammation of the vessel wall [3,4]. This could well be because PR3 is exposed on the surface of quiescent blood neutrophils and/or because it is stored in secretory granules in addition to primary granules [5]. All three NSPs are released into the extracellular medium when neutrophils are activated by cytokines, chemoattractants, or invading pathogens. They may also remain attached to the surface of activated cells as a result of the rapid translocation of intracellular granules [6]. Another peculiar feature of PR3 is the way it is anchored to the surface of the cell membrane of activated neutrophils. Unlike HNE and CG, PR3 is not released from the surface of activated neutrophils by high salt concentrations [7], indicating that its binding is not simply charge dependant [2]. This has been confirmed by the identification of several binding partners [8–10]. Thus, the extracellular distribution of PR3 on neutrophils that have been recruited to and activated at inflammatory sites probably differs from that of other NSPs. We have shown recently that the substrate specificity of PR3 differs from that of HNE in spite of the close structural resemblance between these two proteases [1,2]. Specific sites cleaved by PR3 that resist hydrolysis by HNE have been identified in a variety of physiological protein substrates [11]. This has helped us to understand the specificity of this protease for amino acid residues on either side of its cleavage site,

Abbreviations: PR3, proteinase 3; HNE, human neutrophil elastase; CG, cathepsin G; NSPs, neutrophil serine proteases; α1-PI, alpha-1-antitrypsin; Abz, *ortho*-aminobenzoic acid; (nor)V/(nor)Val, norValine; ANCAs, anti-neutrophil cytoplasmic antibodies; FRET, fluorescence resonance energy transfer; EDDnp, N-(2,4-dinitrophenyl)ethylenediamine; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption ionization; BALF, broncho alveolar lavage fluid.

* Corresponding author. Tel.: +33 2 47 36 62 53.
E-mail address: brice.korkmaz@inserm.fr (B. Korkmaz).

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and to design optimized specific, sensitive PR3 substrates [12]. The pathophysiological role of PR3 in biological fluids or tissue would be better understood if selective inhibitors of this protease were available. And these could be used eventually as therapeutic tools. But all natural inhibitors of PR3 inhibit HNE preferentially [1,13]. While chemical inhibitors including acylating agents, transition state analogs, mechanism-based inhibitors, reversible peptide inhibitors or peptidomimetics have been developed to target HNE, none has been devised that successfully targets PR3 alone [14–17].

Azapeptide protease inhibitors are molecules in which the alpha carbon of the P1 residue has been replaced by a nitrogen atom [18,19]. They retain the main characteristics of their parent peptide and are generally more stable. We have used our knowledge of the substrate specificity of PR3 to modify a specific FRET substrate of PR3 into an azapeptide. Azapeptides generally contain a reactive leaving group, so that they interact with the target protease to form a stable acyl–enzyme intermediate that dissociates very slowly [19]. This is because the substitution of N for the C in the P1 residue decreases the electrophilicity of the P1 carbonyl group and also moves the geometry of the complex away from a tetrahedron. This applies to most of the azapeptides that have been designed to inhibit serine, cysteine and aspartyl proteases [19]. The majority of the azapeptides prepared to inhibit serine proteases are peptide-nitrophenyl esters that release paranitrophenol when the acyl–enzyme complex is formed. These azapeptides can be used to titrate the active sites of proteases because the acyl enzyme complexes are very stable and the interactions obey a 1:1 stoichiometry [20]. However, recently designed azapeptides have a peptide chain on their P′ side bind to the protease non-covalently and reversibly; i.e. without acylation or deacylation [21]. The affinity of their binding to the protease active site is similar to that of the substrate, so that the K_i value is much like the K_m of the parent substrate. FRET substrates interact with proteases on both sides of their cleavage site and the K_m of the interaction is in the micromolar range. We postulated that transforming such a PR3-specific peptide substrate into an azapeptide would result in a competitive inhibitor that specifically inhibited PR3 with a micromolar K_i value.

2. Material and methods

2.1. Materials

Purified PR3 (EC 3.4.21.76) was obtained from Athens Research (Athens, GA) and recombinant PR3 and PR3$K_{99}L$ were obtained as reported earlier [22]. Human neutrophil elastase (EC 3.4.21.37), cathepsin G (EC 3.4.21.20) and α1-PI were obtained from Biocentrum (Krakow, Poland). Chymotrypsin and granzyme B were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Biotin-Ahx-FPR-CMK and Biotin-Ahx-PYFA-CMK were from American Peptide Company (Vista, California, USA) and from Enzyme Systems Products (Solon, Ohio, USA), respectively. Fmoc amino acids were from Advanced ChemTech. Granzyme B substrate Z-IETD-AFC and PR3/HNE substrate Suc-VYDA-pNA were obtained from Merck (Nottingham, UK) and Genecust (Dudelange, Luxembourg), respectively. FRET substrates of PR3 were synthesized as reported [12], or synthesized by Genecust, or Anaspec (Fremont, California, USA) (QXL570-KLPRSARDQK-(5-TAMRA)-NH_2). All other chemical were of analytical grade.

Induced sputum from a patient with severe eosinophilic asthma was collected as described in Ref. [23] and broncho alveolar lavage fluid (BALF) was obtained from a patient with pneumonia using a standard technique [24]. Patients referred to the Department of Pneumology of the University Hospital Center (Tours, France) and gave their informed written consent.

2.2. Synthesis of azapro-3

The Fmoc-Asp(O*t*Bu)-aza(nor)Valine-AlaOH building block was prepared by a seven-step procedure from *tert*-butylcarbazate and L-alanine. 2-Benzyl-aminopropanoate was converted to its isocyanate derivative and reacted with *tert*-butyl 2-propylhydrazinecarboxylate. The amino function was deprotected for coupling Fmoc-Asp(O*t*Bu)OH in the presence of *N*,*N*′-dicyclohexylcarbodiimide and 4,4-dimethylaminopyridine in dichloromethane. The carboxylic acid function was finally removed by hydrogenolysis. All reactions were carried out in an argon atmosphere. Thin layer chromatography was performed using Merck Silica Gel G plates. Materials were detected using ninhydrin, potassium permanganate or phosphomolybdic acid spray reagents. All products were purified by column chromatography and characterized by ^1H and ^{13}C NMR analysis (Brucker 500 MHz).

The azapeptide was synthesized manually using solid phase synthesis on a MBHA resin (2 g) and Fmoc chemistry. Fmoc-Asp(O*t*Bu)-aza(nor)Valine-AlaOH (4 mol eq) was coupled to the resin to which the Tyr_{NO_2}-Gln-Tyr-Asp sequence (1 mol eq) had been coupled previously. The completion of the reaction was checked by a Kaiser test. Alanine and Abz-Valine were finally added using the same procedure and the peptide was released from the resin with ethanedithiol (2.5%) in trifluoroacetic acid (95%) and water (2.5%). The azapeptide Abz-V-A-D-aza(nor)V-A-D-Y-Q-Y_{NO_2} was isolated by ether precipitation, filtered, washed with ether, and characterized by electrospray mass analysis and HPLC on a C18 cartridge. A stock solution of 10 mM azapro-3 in 10% *N*,*N*-dimethylformamide (DMF) was prepared.

2.3. Determination of protease inhibitory activity of azapro-3

2.3.1. Enzyme assays

The activities of free PR3, and HNE were measured in 50 mM Hepes, pH 7.4, 750 mM NaCl, supplemented with 0.05% Igepal CA-630 (v/v). PR3 and HNE were titrated with α1-PI, the titer of which had been determined using bovine trypsin titrated with *p*-nitrophenyl-*p*-guanidinobenzoate. The capacity of azapro-3 (1–100 μM) to inhibit purified PR3 (10–100 nM), PR3$K_{99}L$ and HNE (10–100 nM) was investigated using fluorogenic and chromogenic substrates. The concentration of Suc-VYDA-pNA and its hydrolysis were determined from the absorbance at 410 nm, assuming E_{410} = 8800 M^{-1} cm^{-1} for p-anilide, using a microplate spectrophotometer. The hydrolysis of Abz-peptidyl-EDDnp/Abz-peptidyl-Y_{NO_2} substrates was followed by measuring fluorescence at λ_{ex} = 320 nm and λ_{em} = 420 nm in a microplate fluorescence reader (Spectra Max Gemini; Molecular Devices). The concentrations of the Abz-peptidyl-EDDnp substrate were determined by measuring the absorbance at 365 nm, using $E_{365\ nm}$ = 17,300 M^{-1} cm^{-1} for EDDnp. The substrate QXL570-KLPRSARDQK-(5-TAMRA)-NH_2 was dissolved in water and its hydrolysis was followed by measuring fluorescence at λ_{ex} = 545 nm and λ_{em} = 575 nm. The hydolysis of Z-IETD-AFC (*granzyme B substrate*) was followed by measuring fluorescence at λ_{ex} = 400 nm and λ_{em} = 505 nm.

2.3.2. Inhibition of PR3 in a neutrophil lysate

Purified neutrophils from the blood of a healthy donor (about 10 million cells) were lysed in 500 μL of 50 mM Hepes, pH 7.4, 750 mM NaCl, supplemented with 0.05% Igepal CA-630 (v/v) and centrifuged at 10,000 × *g* to eliminate cell debris and DNA. The proteolytic activity of PR3 in the supernatant were quantified by spectrofluorometry using QXL570-KLPRSARDQK-(5-TAMRA)-NH_2 (2 μM final) and that of HNE using Abz-APEEIMRRQ-EDDnp (3 μM final). The inhibition of PR3 and HNE activities by azapro-3 was measured by incubating each

protease (10 nM) for 5 min at 37 °C with increasing amounts of inhibitor before adding the corresponding FRET substrate.

2.3.3. Inhibition of PR3 in a suspension of purified neutrophils and in crude biological samples

Purified neutrophils (~10 million cells) were activated using the calcium ionophore A23187 (1 μM final) and ~1 million activated cells were incubated with azapro-3 (50–100 μM) for 15 min at room temperature in PBS buffer. The specific PR3 substrate QXL570-KLPRSARDQK-(5-TAMRA)-NH_2 (2 μM) was then added and fluorescence was recorded every 20 s in a microplate fluorescence reader (Spectra Max Gemini; Molecular Devices) under continuous stirring. A 40 μL aliquot of a 10-fold concentrated BALF supernatant from a patient with pneumonia or a sample of crude sputum from a patient with severe asthma were incubated with azapro-3 as above. Because the TAMRA substrate used for this purpose contains two trypsin-like cleavage sites after Arg residues we first incubated the samples with a trypsin-like-directed irreversible chloromethyl ketone inhibitor that does not interfere with PR3 activity. We checked that this inhibitor (Biotin-Ahx-FPR-CMK) did not interfere with PR3 activity as expected from the presence of a P1-Arg residue.

The time course of PR3 inhibition during neutrophil activation was analyzed by recording residual activity on the TAMRA substrate in a suspension of purified quiescent neutrophils during their activation by the calcium ionophore in the presence of azapro-3 (60 μM).

2.4. Chromatographic procedures and peptide analysis

The fluorogenic substrate Abz-VAD(nor)VADYQ-Y_{NO2} (10–30 μM final) or azapro-3 (64 μM final) were incubated with PR3 (100 nM final) at 37 °C for 3 h, 6 h and 24 h in reaction buffer and the proteins precipitated with absolute ethanol (4 volumes). Supernatants containing the hydrolysis products were dried under vacuum and dissolved in 200 μL 0.01% trifluoroacetic acid (v/v), then fractionated by reverse-phase chromatography on a C18 column (2.1 mm × 30 mm, Brownlee, or 2 mm × 33 mm, Uptisphere) at a flow rate of 0.3 mL/min with a linear gradient (0–60%, v/v) of acetonitrile in 0.01% trifluoroacetic acid for 30 or 45 min. Eluted peaks were monitored at 220 nm.

The substrate QXL570-KLPRSARDQK-(5-TAMRA)-NH_2 (2 μM) was added to a neutrophil lysate that had been incubated with 60 μM azapro-3. The supernatant containing azapro-3 and the TAMRA substrate was analyzed as described above. Cleavage sites were identified by MALDI TOF-MS.

2.5. Determination of the molecular mass of the PR3-azapro-3 complex by native mass spectrometry

Experiments were performed on *an ESI*-UHR-*Qq-TOF* (maXis, Bruker Daltonics, Bremen, Germany). For native ESI, PR3 (3 μM final) in 20 mM ammonium bicarbonate, pH 8.0 was incubated with the azapeptide (40 μM final) in the same buffer and the mixture was complemented with 5% acetonitrile. Samples were introduced by direct infusion (flow rate: 1.3 μL/min) with a nebulizer gas pressure of 0.7 bar using a sandwich method. The spray voltage was kept at −4 kV and the dry gas temperature was set to 120 °C. The dry gas flow was increased to 10 L/min to disrupt Igepal CA-630-PR3 clusters. The in-source CID energy was adjusted to 200 eV for optimal detection of high m/z species. The instrument was externally calibrated using hexa (TuningMix®, Agilent Technologies, Santa Clara, CA). Data were processed with Bruker DataAnalysis 4.0.

2.6. Analysis of PR3-azapro-3 interaction reversibility by electrophoresis and western blotting

We assessed the reversibility of the interaction between PR3 and azapro-3 by incubating purified PR3 (100 nM) with azapro-3 (60 μM) for 5 min at 37 °C before adding the irreversible synthetic inhibitor Biotin-Ahx-PYFA-CMK (1 mM final) and incubating this mixture for 30 min to remove azapro-3 from the active site of PR3. For the control, we added the substrate Suc-VYDA-pNA to the initial PR3–azapro-3 mixture to ensure that the inhibition was complete over the time of experiment.

The components of the mixture were separated by SDS-PAGE, 12% NaDodSO$_4$-polyacrylamide gel electroporesis under denaturing conditions, and transferred to a nitrocellulose (Hybond)-ECL membrane. The biotinylated inhibitor was detected using horseradish peroxidase-labeled avidin diluted 1/2500 in PBS-BSA, 5% Tween 0.1% and incubated for 2 h at room temperature after the membrane had been blocked by incubation in PBS 5% BSA for 90 min at room temperature. The membrane was washed and the biotinylated inhibitor revealed by chemiluminescence (ECL Kit).

3. Results

Fig. 1 summarizes the classes of HNE and PR3 inhibitors derived from natural protein inhibitors or synthesized on the basis of their molecular mechanism of action. All but one of them target HNE

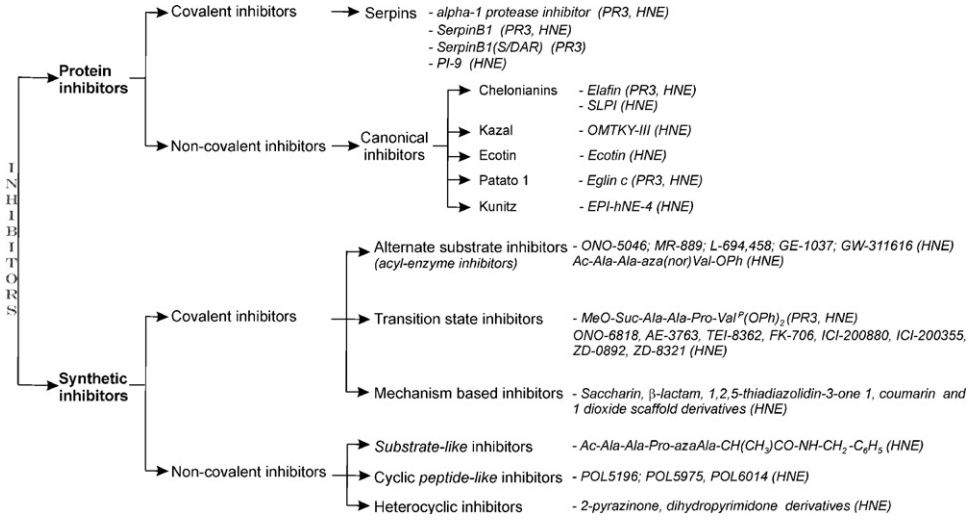

Fig. 1. Inhibitors of human PR3 and HNE.

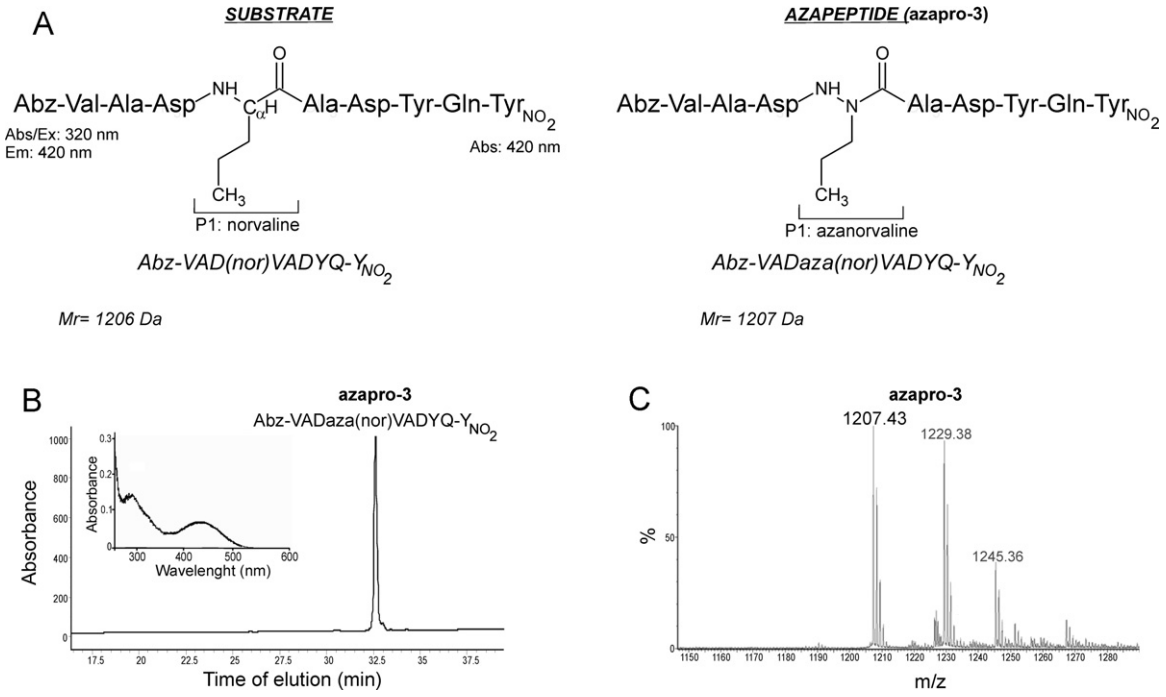

Fig. 2. Structure and purification of the substrate-derived azapeptide, azapro-3. The purity of azapro-3 (A) was checked by HPLC on a C18 cartridge (B) and its Mr was checked by MALDI-TOF MS (C). The insert in (B) shows the absorption spectrum of the purified azapeptide.

preferentially, though several are potent inhibitors of PR3. The exception is a serpin-derived PR3-specific inhibitor recently developed in our laboratory that irreversibly inhibits PR3 and clears the active protease from the surface of triggered neutrophils [22].

3.1. *Synthesis of* Abz-VADaza(nor)VADYQY$_{NO_2}$

We have previously prepared FRET substrates that were specifically cleaved by PR3 with K_m values in the micromolar range. We took advantage of the high affinity of these substrates to

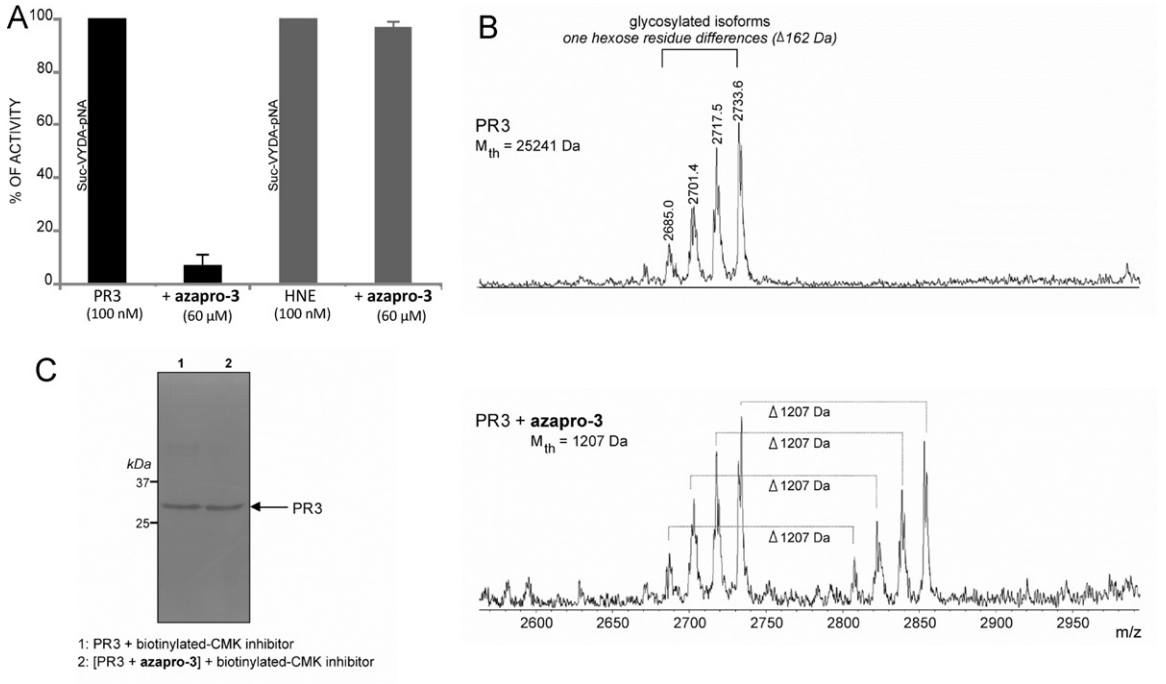

Fig. 3. (A) Residual activity of purified PR3 and HNE (100 nM) incubated with azapro-3 (60 μM final) for 5 min. (B) ESI mass spectra of recombinant PR3 and the PR3–azapeptide complex in 20 mM ammonium bicarbonate, pH 8.0. The 10^+ charge state is shown. (C) Western blotting analysis of the reversibility of the PR3-azapro-3 complex. The PR3-azapro-3 inactive complex was incubated for 1 h with a biotinylated irreversible inhibitor of PR3 (Biotin-Ahx-PYFA-CMK) and the mixture was analyzed by western blotting and revealed by avidin. The biotinylated inhibitor displaced azapro-3 from its complex with PR3.

render their scissile bond resistant to cleavage while keeping their affinity and selectivity towards the target protease. For this purpose we selected one of these substrates (Abz-VAD(nor)VADYQY$_{NO_2}$, $K_m = 1.2\ \mu M$) which is specifically cleaved by PR3 in homogenates of neutrophil/monocytes extracts, BALFs and sputa and replaced the alpha carbon of the P1 residue (norVal) by a nitrogen atom. We anticipated that the resulting azapeptidyl construct should have the same properties of resistance to proteolysis and oxidation as the parent substrate. The aza function was introduced by constructing a Fmoc-derivative of a building block (Fmoc-Asp-(aza)norVal-Ala) as reported under Section 2 and we used it for manual peptide synthesis. The peptide sequence and the fluorescence donor/acceptor pair (Abz/Tyr$_{NO_2}$) of the parent substrate were conserved to preserve interactions within the active site and to favor water solubility of the molecule (Fig. 2A). The azapeptide, provisionally called azapro-3, was purified by HPLC and analyzed by MALDI-TOF MS (Fig. 2B and C). Because of the close structural relationship between azapro-3 and its parent substrate and the possible interference with the quencher group of the inhibitor, no PR3 substrate with an Abz fluorophore can be used for functional assays. We encountered this limitation by developing a specific FRET substrate of PR3 linked to a red fluorochrome (TAMRA) (QXL570-KLPRSARDQK-(5-TAMRA)-NH$_2$), the spectrum of which does not overlap with that of Abz. Further, this sequence which derives from that of pro-interleukin 8 is cleaved by PR3 but not by HNE and CG and the K_m for its hydrolysis is about 4 times higher (K_m = 4.5 ± 0.5 μM) than that of Abz-VAD(nor)VADYQY$_{NO_2}$, avoiding a possible competition with the inhibitor. We also used a chromogenic peptide-paranitroanilide substrate of both HNE and PR3 (Suc-VYDA-pNA) for kinetic measurements.

3.2. Azapro-3 inhibitory activity and mechanism of action against PR3

We first assayed the capacity of the azapeptide, azapro-3, to inhibit recombinant human PR3 and HNE. In addition we tested other chymotrypsin-like proteases including CG, chymotrypsin, and granzyme B. All proteases (100 nM) were incubated with azapro-3 (20–100 μM) for 15 min at 37 °C in appropriate buffer. About 90% of PR3 was inhibited, less than 5% HNE activity was inhibited (Fig. 3A), while CG and other chymotrypsin-like proteases were not inhibited (not shown). We then determined whether the azapeptide was a reversible inhibitor, or whether it formed a stable acyl–enzyme complex upon cleavage of the P1–P1′ bond. Formation of the acyl–enzyme complex would result in the release of the C-terminal fragment of the inhibitor and the appearance of fluorescence on the complex. We used a micromolar concentration of PR3 mixed with a 10-fold molar excess of

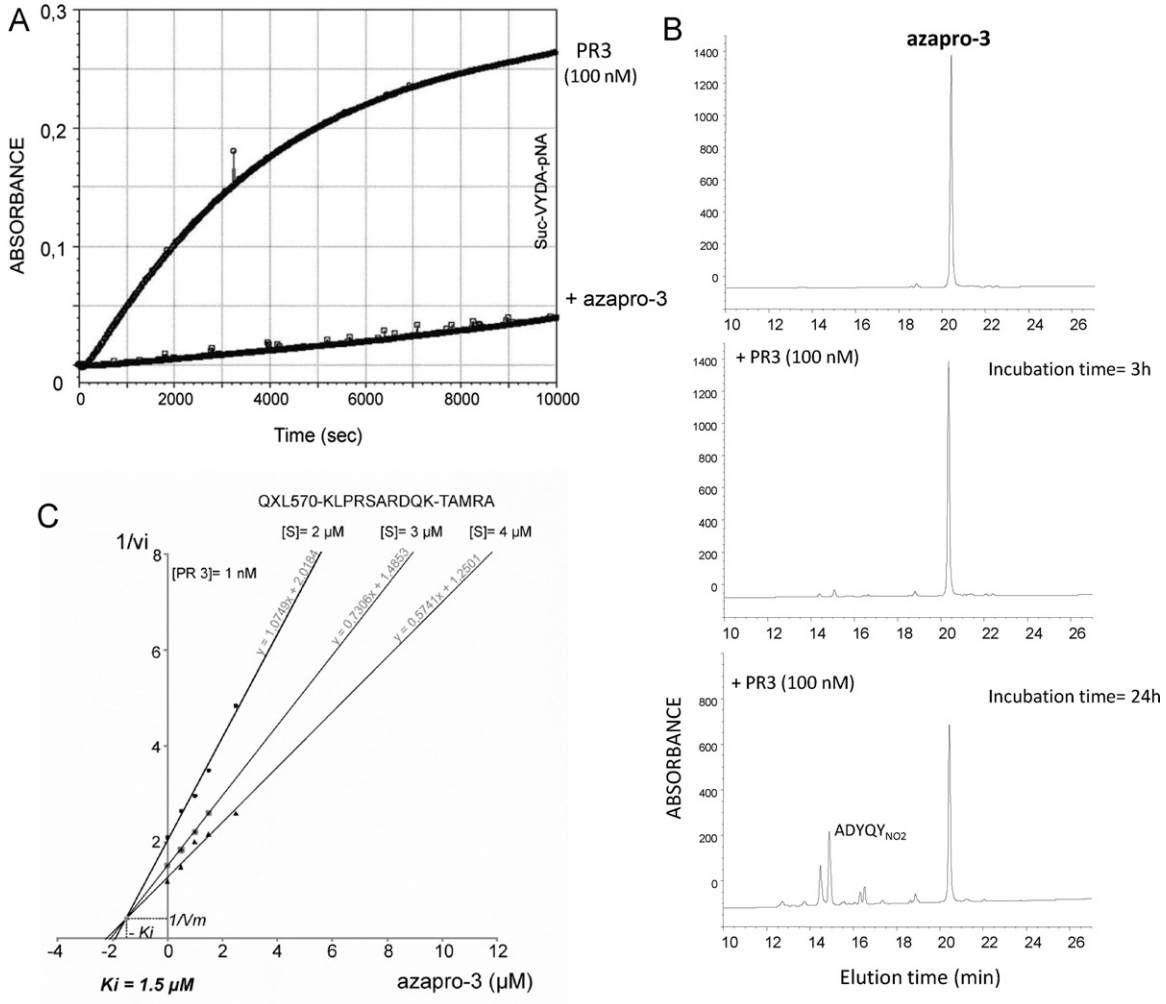

Fig. 4. (A) Stability of PR3 inhibition over time. Experimental conditions were the same as in Fig. 3(A) and the reactions were all started by adding Suc-VYDA-pNA as a substrate and recording absorbance at 410 nm. (B) Azapro-3 was partially broken down only after incubation with 100 nM PR3 for 24 h. The cleavage site was the aza(nor)V–A bond, i.e. the same position as that in the parent substrate. (C) K_i determination using the Dixon plot. The rate of hydrolysis of the fluorescent PR3 substrate QXL570-KLPRSARDQK-TAMRA was measured using increasing concentrations of azapro-3 at three substrate concentrations. The curves obtained show competitive inhibition with a K_i of 1.5 μM (mean of 3 independent experiments), close to the K_m of the parent substrate.

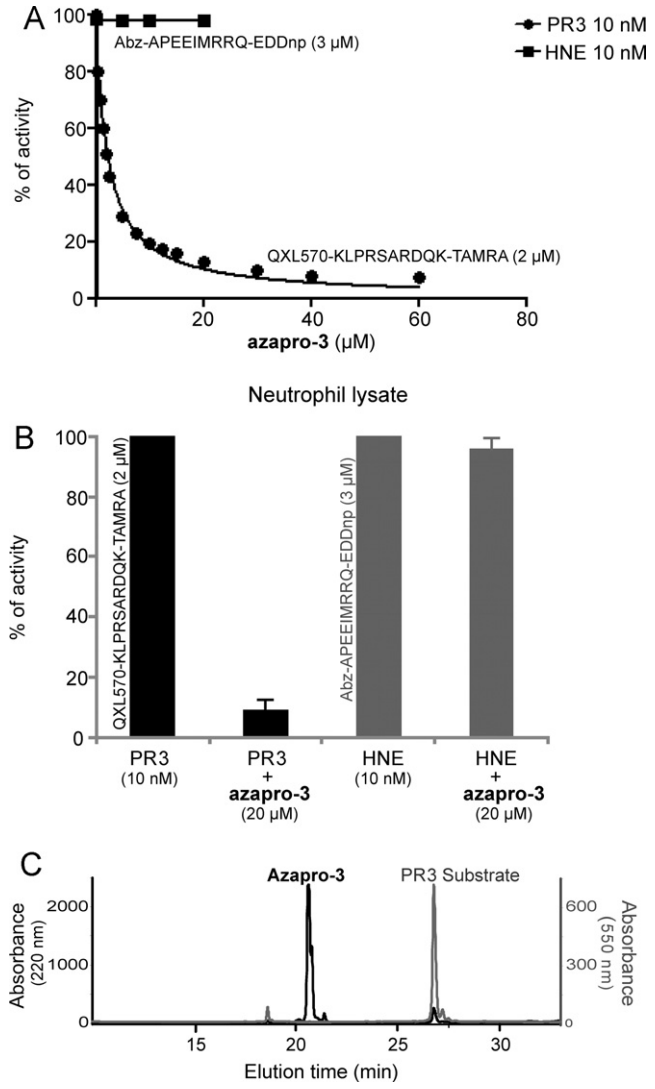

Fig. 5. (A) Residual activity of purified PR3 and HNE (10 nM) after incubation for 5 min at 37 °C with increasing amounts of azapro-3. (B) Residual activity of PR3 and HNE (10 nM) in a neutrophil lysate incubated with 20 μM azapro-3. (C) Stability of azapro-3 (black line) and the PR3 TAMRA substrate (grey line) after incubation with the neutrophil lysate plus azapro-3. The mixture was precipitated with ethanol and analyzed by HPLC. There was no significant proteolytic breakdown of azapro-3 and no hydrolysis of the PR3 substrate, again demonstrating that PR3 can be inhibited in a protease-rich environment.

azapeptide to optimize detection of peptide fragments and fluorescence. There was total inhibition of PR3 immediately after mixing enzyme and inhibitor but no released inhibitor C-terminal fragment was found by HPLC, and the complex did not fluoresce as it should have if an acyl enzyme complex had formed (not shown). This strongly suggests that azapro-3 is a reversible inhibitor that remains structurally intact upon interaction with PR3. The formation of a non-covalent PR3–azapro-3 complex was confirmed by native mass spectrometry through direct measurement of the Mr of the complex, showing a 1207 Da mass increase compared to PR3 which corresponds to the mass of azapro-3 (Fig. 3B). Western blotting also showed that azapro-3 was easily removed from the complex by a biotinylated irreversible chloromethylketone inhibitor, which confirms the reversibility of the interaction (Fig. 3C). Azapeptides are more resistant to proteolysis than their parent peptides because of the reduced flexibility imposed by replacing the C with N [19]. But the absence of cleavage of azapro-3 during its interaction with the protease does not mean that it resists proteolysis by its target enzyme or other proteases that may be in a biological environment. Some azapeptides are rapidly broken down, and this seriously impairs their use *in vivo* [25]. We controlled that inhibition remained stable over a 3 h period of time (Fig. 4A) and that no cleavage of azapro-3 occurred during that time (Fig. 4B) while the parent FRET substrate was almost entirely hydrolyzed in these conditions (not shown). Azapro-3 was partially broken down only after incubation for 24 h. It was cleaved at a single site, the aza(nor)Val–Ala bond, i.e. at the same P1–P1′ site as the parent substrate (Fig. 4B).

We calculated the K_i for the interaction of azapro-3 with purified PR3 by incubating constant amounts of PR3 with increasing amounts of inhibitor at different substrate concentrations. The Dixon plot ($1/v_i = f(I)$) shows that inhibition is purely competitive with a K_i of 1.5 μM (Fig. 4C). As expected this value is close to that of the K_m calculated for the model substrate. This indicates that the N_α for C_α substitution in the P1 residue without any other change in the model substrate does not significantly impair accommodation of the peptide sequence within the active site. We confirmed that the interaction with azapro-3 was not significantly different from that with the parent substrate using recombinant PR3 with a single Lys/Leu mutation within the S2 subsite. The K_i was increased 10-fold using PR3$K_{99}L$, as was the K_m for the interaction between the recombinant protease and Abz-VAD(nor)VADYQ-EDDnp, indicating that the P2 Asp in the substrate and in the inhibitor occupy the same position within the active site of PR3.

3.3. Selective inhibition by azapro-3 of PR3 in neutrophil-containing suspensions and homogenates

Any use of azapro-3 to target PR3 in a pathophysiological environment must also assume that no other protease is present that would rapidly degrade the inhibitor and thus impair its function. While HNE is not inhibited by azapro-3, it could destroy the inhibitor at a significant rate. We compared the inhibition of 10 nM PR3 in solution by 20 μM azapro-3 with that of a whole neutrophil lysate with the same PR3 activity. PR3 was ~90% inhibited after incubation for two min in both situations (Fig. 5A and B) and this remained the same when the neutrophil lysate–inhibitor mixture was incubated for 30 min before adding the substrate. HPLC analyses indicated that both the PR3 substrate and azapro-3 remained undegraded (Fig. 5C). Thus azapro-3 remains fully active and stable in a high-protease reaction mixture. Because PR3 lies in part at the surface of activated neutrophils, the next question was how does azapro-3 affect membrane-bound PR3.

The PR3 at the surface of triggered neutrophils retains its enzymatic activity. It hydrolyzes the PR3-specific peptide substrate Abz-VAD(nor)VADYQ-EDDnp and can be inhibited by irreversible peptide inhibitors and protein inhibitors [26]. We first checked that azapro-3 inhibited PR3 in a suspension of activated neutrophils (Fig. 6A) and that it was neither broken down nor internalized by activated cells (Fig. 6B). Because it is a reversible inhibitor, we looked at the effect of PR3 binding to the membrane on the equilibrium between free and bound protease, thus making the inhibition less efficient. Quiescent neutrophils were triggered with the calcium ionophore A23187 in the presence or absence of azapro-3 (60 μM final) and the PR3 activity was measured continuously. PR3 activity increased significantly as soon as the ionophore was added to the quiescent neutrophils, but no activity was detected when the ionophore was added to cells that had been incubated with 60 μM azapro-3, indicating that PR3 was fully inhibited by azapro-3 as soon as it was secreted by activated neutrophils (Fig. 6C). However we showed by flow cytometry that PR3–azapro-3 complexes remained at the

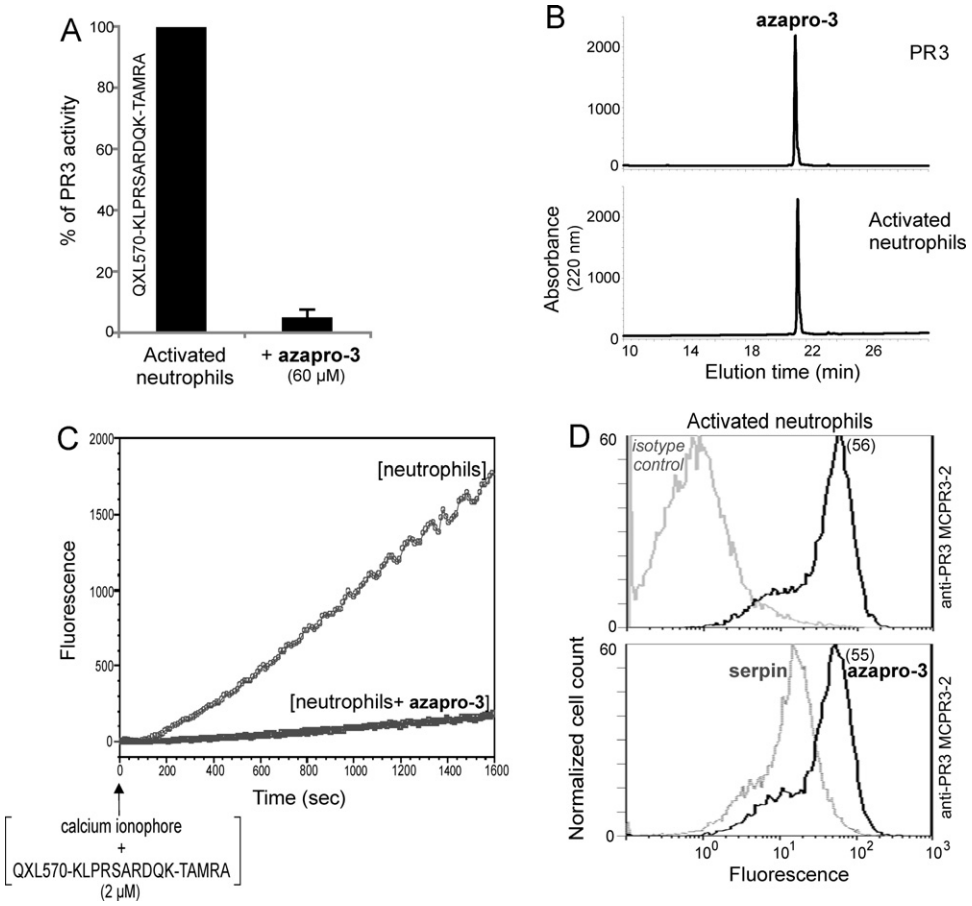

Fig. 6. (A) Inhibition of PR3 by azapro-3 after 15 min in a suspension of activated neutrophils (~10^6 cells). (B) HPLC profile of azapro-3 (60 μM) after a 15 min incubation time with purified PR3 (top) and with activated neutrophils (down) showing the stability of the inhibitor in the presence of activated cells and the absence of internalization of azapro-3 by activated neutrophils. (C) Time course of inhibition of PR3 in a suspension of purified neutrophils during their activation by the calcium ionophore A23187: quiescent neutrophils were incubated with azapro-3 before adding a mixture of PR3 substrate and calcium ionophore. (D) Flow cytometry analyses of membrane-bound PR3 on A23187-activated neutrophils. Neutrophils were labeled with the anti-PR3 MCPR3–2 mAb revealed by FITC-conjugated anti-mouse IgG before (top panel) and after (bottom panel) incubation with azapro-3 (dark line) and a PR3 inhibitory serpin (α1-PI). The serpin but not azapro-3 has removed most of PR3 from the cell surface.

neutrophil surface. This differs from those formed using serpin-like inhibitors that were cleared from the cell surface (Fig. 6D) [22,26].

Quiescent neutrophils produced little or no PR3 activity, which confirms that constitutive membrane-bound PR3 is inactive [26]. Trypan blue exclusion tests also showed that incubating azapro-3 with neutrophils for 4 h did not change cell viability significantly (not shown).

If azapro-3 is to be used to inhibit the PR3 activity in an inflammatory biological fluid such as the lung secretions of patients with inflammatory lung diseases associating the recruitment of neutrophils, the inhibitor must remain active as a PR3 inhibitor in these conditions. We tested this using a biological sample in which neutrophils accounted for only 20–30% of the total cells, so that PR3 was not a prominent element in the crude sample. We checked that this sample (induced sputum from an asthmatic patient) cleaved the PR3 selective substrate Abz-VAD(nor)VADYQ-EDDnp at the expected scissile bond ((nor)V-Ala) (Fig. 7A) and used western blotting with an anti-PR3 antibody to show that free PR3 was present in the sample (Fig. 7B). The rate of substrate hydrolysis indicated that the concentration of active PR3 was in the nanomolar range. The PR3 activity in the crude sample was >90% inhibited by incubation with azapro-3 (60 μM final) for 15 min which confirms that the inhibitor remains efficient even in a heterogeneous biological sample where its target is not a major component. Inhibition remained stable for at least 2 h after azapro-3 had been added (Fig. 7C). The same result was obtained using a concentrated BALF supernatant from a patient with pneumonia.

4. Discussion

Human NSPs are potential targets for anti-inflammatory treatment, but their individual contributions to the development of inflammatory diseases are not fully understood. This is partly because it is difficult to measure individual proteolytic activities in a complex biological medium and to target individual proteases using natural or synthetic inhibitors. This is especially true for HNE and PR3 that are structurally and functionally very similar. We previously developed sensitive FRET substrates bearing an N-terminal ortho-aminobenzoyl (Abz) fluorescent group and a C-terminal quenching group (N-(2,4-dinitrophenyl) ethylenediamine (EDDnp) or nitroTyr) as a donor/acceptor pair, that fully discriminate between HNE and PR3 activities [12]. The peptide moieties of these substrates were adapted from the sequence of natural substrates or from the inhibitory loop of natural inhibitors of these proteases.

The residues at the cleavage site of peptide substrates or protein inhibitors have been modified to slow down or totally hinder cleavage of the scissile peptide bond and thus turns a substrate into an inhibitor. We recently produced a recombinant serpinB1, a polyvalent inhibitor of neutral serine proteases, that was mutated within its reactive center loop to make it an irreversible, specific

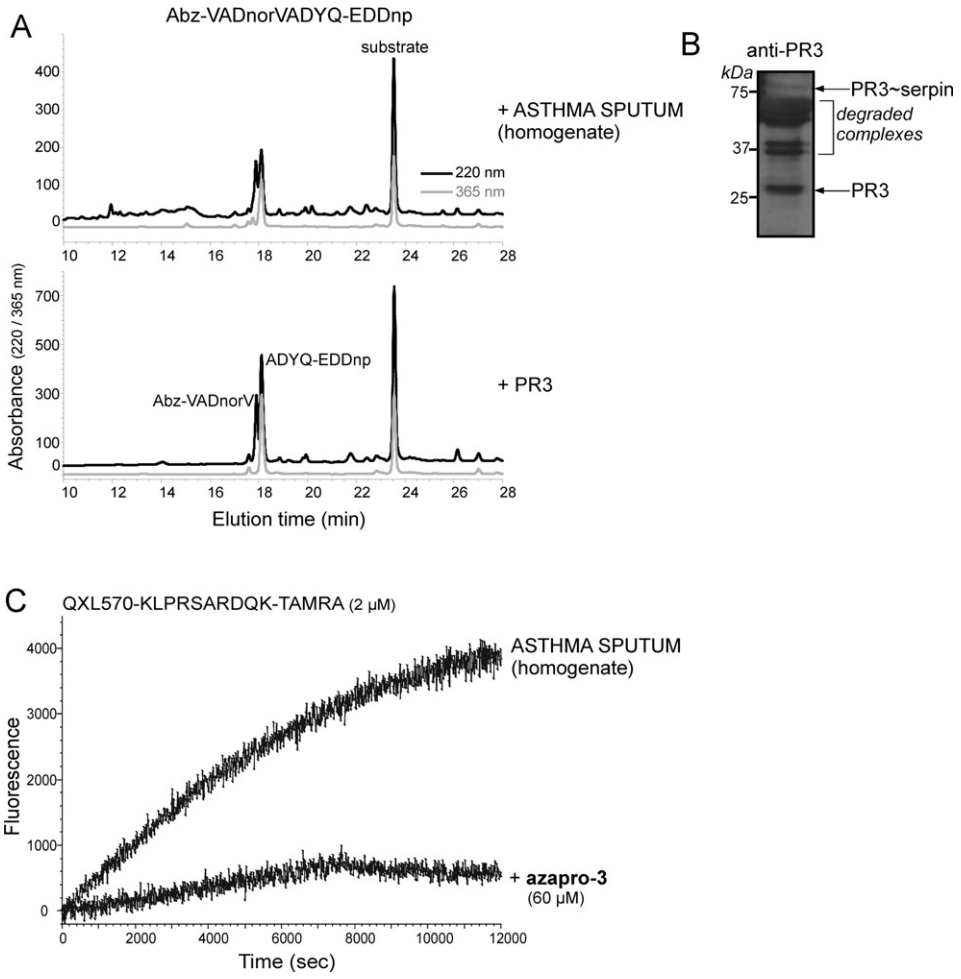

Fig. 7. (A) HPLC profiles of hydrolysis products of Abz-VAD(nor)VADYQ-EDDnp substrate after incubation with an asthma sputum homogenate and purified PR3. (B) Western blotting of asthma sputum using anti-PR3 antibodies confirmed the presence of free PR3 (Mr ~28 kDa) and serpin-bound PR3 (Mr ~75 kDa). (C) Time course of PR3 activity in asthma sputum before and after incubation with azapro-3.

inhibitor of PR3 [22]. This protein inhibits both soluble and membrane-bound PR3 by forming soluble complexes, and can help to control neutrophil activation by anti-PR3 antibodies, such as occurs in Wegener's granulomatosis [1,27]. Chemically modified small peptides however are more readily produced in larger amounts than large Mr serpins; they are easier to handle and may be more appropriate for treating inflammatory diseases that involve neutrophil recruitment. Peptidomimetics retain the main function of the parent peptide and generally have better bioavailability and metabolic stability, which makes them good candidates for therapeutic use [19]. The substitution of C for N in the P1 residue induces a loss of chirality and reduces the flexibility of the parent peptide, giving it unique conformational properties. Nevertheless, the conformation adopted by the aza amino acid moiety around the nitrogen may be significantly affected by the surrounding peptide sequence [28], so that it is impossible to anticipate on the inhibitory properties of the azapeptide. Serine and cysteine proteases generally interact with azapeptides to form stable acyl–enzyme complexes and release the C-terminal end of the inhibitor [20,29]. But azapeptides with a peptide C-terminal extension beyond the P1 residue may behave as reversible uncleaved inhibitors that remain in equilibrium with the protease [21,30]. This precludes titration, but predicts a K_i comparable to the K_m of the original substrate. Such a mechanism has been demonstrated using 9- to 18-mer substrate-based azapeptides of the hepatitis C virus NS3 serine protease [21]. We used a PR3-specific FRET substrate (Abz-VAD(nor)VADYQY$_{NO2}$) with a low K_m (μM) and changed the alpha carbon of the P1 norVal residue to a nitrogen. We assayed the capacity of the resulting azapeptide, azapro-3, to inhibit PR3. But measuring PR3 activity in the presence of azapro-3 requires a specific fluorescent substrate with an emission spectrum different from that of Abz. We have developed a TAMRA-containing substrate (excitation/emission wavelength of 540/575 nm) and used it to demonstrate that azapro-3 is a competitive, reversible inhibitor that is not cleaved during its interaction with PR3. The K_i was in the micromolar range, as expected, and the inhibitor did not inhibit the related neutrophil proteases HNE and CG, in keeping with the properties of the parent substrate.

An azapeptide containing a fluorophore/quencher pair is useful for demonstrating its stability in biological samples containing other proteolytic enzymes, since fluorescence would be detected as soon as any peptide bond is cleaved within the peptide backbone. We observed no fluorescence of Abz when azapro-3 was added to a lysate of neutrophils, or to the lung secretions of patients with pulmonary inflammatory diseases, which demonstrates the stability of azapro-3 and further validates the mechanism of inhibition. Azapro-3 was significantly degraded only after it had been incubated with PR3 for at least 24 h. A single cleavage site was observed at the P1–P1′ site in these conditions. This degradation was so slow that it did not interfere with the almost immediate inhibition that occurred with inhibitor

concentrations in the 10-micromolar range. Lastly, azapro-3 did not alter the viability of a suspension of purified neutrophils, even after incubation with the inhibitor for several hours.

In summary, we have shown that this azapeptide selectively targets PR3 even in complex biological samples containing a variety of proteases. This will certainly help to elucidate the biological function of PR3, whose activity is hard to distinguish from that of HNE. This explains why all the PR3 inhibitors described until recently preferentially target HNE. We recently engineered a recombinant form of serpinB1 that selectively targets PR3 [22] azapro-3 has the advantage of being a reversible inhibitor, which overcomes the main concerns about irreversible inhibitors, such as the immunogenicity of covalently modified proteins and the consequences of suboptimal inhibition during long term trials [31]. Last, azapro-3 is a peptide-derived inhibitor that strongly resists proteolytic degradation.

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