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In Silico Design, Synthesis, and Assays of Specific Substrates for Proteinase 3: Influence of Fluorogenic and Charged Groups

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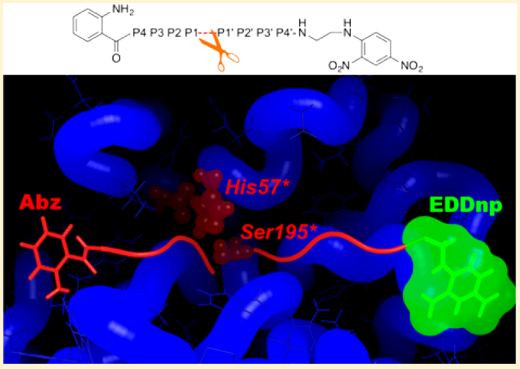
Supporting Information

ABSTRACT: Neutrophil serine proteases are specific regulators of the immune response, and proteinase 3 is a major target antigen in antineutrophil cytoplasmic antibody-associated vasculitis. FRET peptides containing 2-aminobenzoic acid (Abz) and *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) as fluorophore and quencher groups, respectively, have been widely used to probe proteases specificity. Using in silico design followed by enzymatic assays, we show that Abz and EDDnp significantly contribute to substrate hydrolysis by PR3. We also propose a new substrate specific for PR3.



INTRODUCTION

Neutrophil serine proteases (NSPs) proteinase 3 (PR3, EC 3.4.21.76) and human neutrophil elastase (HNE) are therapeutic targets in a number of chronic inflammatory diseases.[1] In particular PR3 is a major target antigen in antineutrophil cytoplasmic antibody (ANCA) associated vasculitis, a life-threatening condition for which molecular mechanisms remain to be uncovered. Moreover the role of each of the three NSPs in diseases and inflammation is only emerging and there is a need for specific substrates that can be used for in vitro and cellular assays.[2] Rational design of specific peptides is also a natural step toward the design of druggable low-molecular weight compounds. Achieving specific targeting of either of these proteases is challenging, as the mature forms of PR3 and HNE share a high sequence identity (56%) and structural similarity.[3] We and others have shown that differences between PR3 and HNE in the nature of the S2, S1′, S2′, and S3′ substrate binding sites (Schechter convention[4]) can be exploited to design specific substrates for PR3.[5,6] Because the charged amino acids Lys99 (S2), Asp61 (S1′ and S3′), and Arg143 (S2′) have polar or hydrophobic counterparts in HNE (Asn, Leu, and Leu, respectively), peptides with negatively charged residues at P2 and P2′ and positively charged residues at P1′ and P3′ are highly specific for PR3.[5,6] Most recent investigations of PR3 specificity have been conducted using fluorescence resonance energy transfer (FRET) peptide substrates containing N-terminal 2-aminobenzoic acid (Abz) as a fluorophore and C-terminal *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) as a quencher.[7,8] These groups have been instrumental for the investigation of the proteolytic hydrolysis of substrates with sequences extending on both sides of the cleavable bond. Yet their use introduces a limitation on the substrate concentration that can be used for enzymatic assays to avoid inner-filter effect arising from intermolecular quenching. Moreover, since PR3 and HNE have extended substrate binding sites, the terminal groups might bind to the enzyme and possibly influence enzyme activity. We have used a combination of in silico design, peptide synthesis, and enzymatic assays to investigate (i) the influence of the FRET groups Abz/EDDnp on the hydrolysis of octapeptide substrates by PR3 or HNE and (ii) the effect of charged amino acids at P2, P2′, and P3′. The use of high performance liquid chromatography (HPLC), unlike fluorescence measurements, allows us to compare the cleavage efficiency of the enzyme for substrate sequences with and without the FRET groups and work at higher substrate concentration than reported in previous studies.

RESULTS AND DISCUSSION

Substrate Design and Modeling. We have earlier demonstrated that the number and strength of the enzyme−substrate interactions for a given peptide in the Michaelis complexes of PR3 (and HNE) constituted a good predictor of the ability of the enzyme to efficiently cleave that substrate.[6] We here first investigate the interactions of PR3 and HNE with a peptide, Abz-VADnVADYQ-EDDnp (nV = norvaline), reported to be specific for PR3.[5,8] We used molecular docking

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and molecular dynamics (MD) simulations to build structural models of the Michaelis complexes and further analyzed the MD trajectories by free energy decomposition to quantify the relative contribution of the different chemical groups to the overall binding affinity and the energetic costs of replacing key amino acids. Another advantage of MD simulations over static structural considerations or rigid molecular docking is that it takes into account the interdependence between pockets and their plasticity.[6] We then varied the nature of the amino acids at P2, P2′, and P3′, as well the N- and C-terminal groups (presence or absence of Abz and EDDnp). Herein, the substrates will be referred to depending on the nature of P2-P2′P3′: VA**D**nVA**DY**Q-NH_2 as D-DY and Abz-VA**D**nVA**DY**Q-EDDnp as D-DY_{FRET}, etc. Detailed results of the MM/PBSA free energy decomposition are given as Supporting Information (Table S1 and Figures S1−S3).

The simulations reproduce the expected structural features for chymotrypsin-like serine proteases including the antiparallel β-sheet between backbones of the P1-P3 residues and of amino acids 214−216. Moreover, as expected, the side chain of P1-nVal mediates extensive hydrophobic contacts with residues of the S1 site. Interestingly the simulations of PR3 complexed with the fluorogenic substrates show that the EDDnp and Abz groups are in contact with the enzyme. EDDnp essentially interacts with the loop Met35-Ser39 (side chains of Met35 and Asn38A, backbones of Pro38B and Gly38C), while Abz interacts with Trp218 (a glycine in HNE). Although the loop Met35-Ser39 is shorter in HNE than PR3, most interactions mediated by EDDnp are conserved between PR3 and HNE. The free energy decompositions of the five complexes between PR3 and the FRET substrates show that the two groups are involved in van der Waals interactions. In particular EDDnp exhibits binding energies with PR3 (−6.6 to −4.6 kcal/mol) comparable to that of the P1 side chain (nVal) (from −5.8 to −5.5 kcal/mol), which is the side chain of the substrates with the highest contribution. The simulations indicate that EDDnp and Abz contribute to the stability of the Michaelis complexes, thus leading to tighter complexes than with the substrates not containing the fluorogenic groups.

The MD simulations show a favorable contribution of P3′ to the interaction between HNE and P3′-Tyr (−3.4 ± 2.7 kcal/mol) in D-DY_{FRET}, which indicates that D-DY_{FRET} might be able to significantly bind to HNE. The corresponding P3′ contribution in PR3/D-DY_{FRET} is also favorable (−0.7 ± 1.5 kcal/mol). To improve the specificity of the substrate for PR3, we replaced the tyrosine by an arginine (P3′-Arg in D-DR_{FRET}), yielding as expected an unfavorable P3′-S3′ interaction with HNE (4.0 ± 3.8 kcal/mol) but a favorable one with PR3 (−1.8 ± 4.4 kcal/mol). In D-DR_{FRET} the three sites (S2-S2′S3′) are charged amino acids and engage in electrostatic interactions with PR3 and hydrogen bonds with K99, R143, and D61, respectively (Figure 1). We then exchanged the charged P2-Asp for a neutral but polar amino acid: asparagine (P2-Asn in N-ER_{FRET}). The only significant change in energy upon this modification is a moderate favorable increase of the van der Waals contribution of P2-Asn in PR3 and HNE, indicating that the N-ER sequence could bind preferentially to PR3 compared to HNE. Unfortunately a small hydrophobic amino acid in P2 (P2-Ala in A-ER_{FRET}) does not contribute any unfavorable contribution in HNE and thus might increase the overall binding affinity for the latter. We next proceeded to synthetize these five sequences (with and without EDDnp, resulting in 10 substrates) and experimentally investigate their hydrolysis by PR3 and HNE.

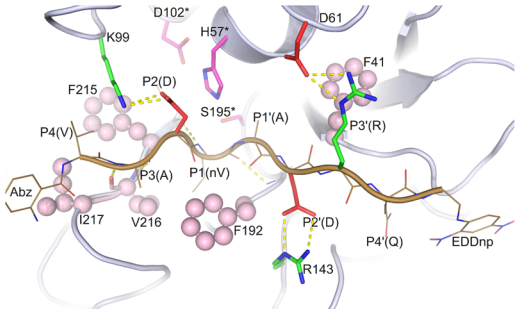


Figure 1. Structural model of the D-DR_{FRET}/PR3 Michaelis complex from MD simulations: PR3 in gray, peptide backbone in ochre, catalytic triad in magenta, charged residues in green or red; pink spheres, PR3 residues mediating the strongest van der Waals interactions.

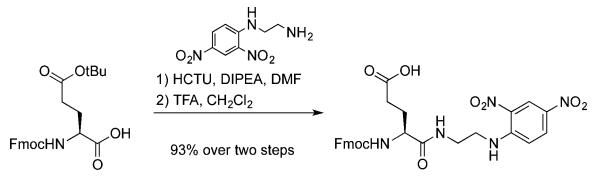
Synthesis. All substrates listed in Table 1 were then synthesized using Fmoc solid phase peptide synthesis. EDDnp was incorporated into the substrates by coupling of Fmoc-Glu-EDDnp to a Rink amide resin through the side chain carboxylic acid, which ensured formation of the desired glutamine side chain upon cleavage of the peptides from the solid support. The required Fmoc-Glu-EDDnp was prepared (Scheme 1) by HCTU-mediated coupling of Fmoc-Glu(O^tBu)-OH with *N*-(2,4-dinitrophenyl)ethylenediamine, which in turn was prepared by reacting ethylenediamine dihydrochloride with 2,4-dinitrochlorobenzene in refluxing ethanol. Subsequent deprotection of the *tert*-butyl ester using TFA gave the desired material in 93% yield over the two steps. In addition to the substrates listed in Table 1, we also synthetized substrates containing two variants of the VA**D**nVA**DY**Q-NH_2 sequence,

Table 1. Percentage of Hydrolysis of Substrates by PR3 and HNE Measured by HPLC[a]

peptide name (P2-P2′P3′)	PR3		HNE	
	H/NH_2[b]	FRET[c]	H/NH_2[b]	FRET[c]
D-DY	4.8 ± 1.3	99.7 ± 0.1	1.6 ± 1.2	52.8 ± 5.7
D-DR	nh[d]	93.4 ± 2.7	nh[d]	7.6 ± 2.6
N-ER	nh[d]	99.2 ± 0.4	nh[d]	10.9 ± 2.4
N-EY	0.9 ± 0.9	94.2 ± 3.7	3.4 ± 1.4	75.5 ± 10.0
A-ER	0.7 ± 1.2	99.9 ± 0.1	nh[d]	66.1 ± 2.2

[a]Peptide names reflect the amino acids at P2, P2′, and P3′. The remaining amino acids in the sequence are identical for all peptides (Val-Ala-**P2**-nVal-Ala-**P2′**-**P3′**-Gln). Values reported are the mean and standard deviation of three experiments. [b]Results for peptides with a free N-terminal amine and a C-terminal amide. [c]Fluorogenic substrates (Abz-sequence-EDDnp). [d]nh, not hydrolyzed; no product could be detected.

Scheme 1. Synthesis of Fmoc-Glu-EDDnp



either with only Abz (Abz-VA**D**nVA**DY**Q-NH_2, D-DY_{Abz}) or only EDDnp (VA**D**nVA**DY**Q-EDDnp, D-DY_{EDDnp}).

Influence of the FRET Groups on Substrate Hydrolysis. The percentage of hydrolysis of each peptide by PR3 and by HNE in vitro was measured using HPLC analysis of the reaction products (Table 1). We also analyzed the products resulting from reactions of each fluorogenic substrate with PR3 and HNE (when relevant) using liquid chromatography−mass spectrometry (LCMS) and found that the expected products were formed upon hydrolysis of FRET substrates by both enzymes (cleavage between P1-nVal and P1′-Ala). However, HNE yielded additional hydrolysis products (cleavage between P1′-Ala and P2′, detailed in Supporting Information).

The fluorogenic substrates were all more extensively hydrolyzed by PR3 and HNE than the nonfluorogenic substrates. None of the five substrates without Abz/EDDnp showed a significant degree of proteolysis by PR3 or HNE under the experimental conditions. This difference is in agreement with the predictions from MD simulations and free energy decompositions. Interestingly adding EDDnp only to the D-DY sequence (D-DY_{EDDnp}) increased the percentage of hydrolysis by PR3 (79.5 ± 9.7%) while 99.8 ± 0.2% of D-DY_{Abz} is cleaved. This indicates an equal contribution of the fluorophore and the quencher to the increased hydrolysis, although the simulations predicted a stronger effect of EDDnp compared to Abz (cf. Table S1 in Supporting Information). This discrepancy could be a consequence of the fact that the simulations, by considering only the interactions in the Michaelis complexes, miss the importance of interactions in the reaction intermediates. Moreover it is important to consider that strong interactions might stabilize the Michaelis complex or reaction intermediates and thereby influence the activation energy of the corresponding reaction step.

Hydrolysis of D-DY_{FRET} (10 μM) by PR3 (5 nM) was slowed by a factor of 2 in the presence of an excess of D-DY (200 μM) compared to the same experiment with only D-DY_{FRET} (data not shown), indicating that the unmodified peptide does bind to PR3 and to the same binding sites as its FRET counterpart, albeit with a lower affinity. This result further confirms that the Abz and EDDnp groups contribute to the hydrolysis rate of the substrates by PR3 and HNE.

Influence of P2, P2′, and P3′ on Substrate Hydrolysis. In what follows, we focus on the fluorogenic peptides. The percentages of hydrolysis reported in Table 1 show that two of the five FRET substrates are preferentially cleaved by PR3. D-DR_{FRET} is cleaved by PR3 (93.4 ± 2.7% hydrolysis) but hardly by HNE (7.6 ± 2.6%). N-ER_{FRET}, the result of our attempts to reduce the number of charged amino acids in the substrates, is also preferentially cleaved by PR3 with a measured percentage of hydrolysis of 99.2 ± 0.4% and only 10.9 ± 2.4% hydrolysis by HNE. Although these differences indicate only a moderate selectivity, it is important to keep in mind that these results are obtained at high enzyme concentrations in order to detect even low level of hydrolysis of the non-FRET substrates.

Interestingly the substrate D-DY_{FRET} has been earlier reported to be specific for PR3,[5] in a study where rather low substrate concentrations (below 1 μM) were used for the fluorescence activity assays. In the present work, using higher substrate concentrations (90 μM, near saturation; see Table 2), we observe a significant cleavage of D-DY_{FRET} by HNE (52.8 ± 5.7%), in agreement with MD simulations. This discrepancy could be due to the difference in the experimental conditions between the two studies. Attempts to replace P2-Asp by a small nonpolar alanine (cf. A-ER_{FRET} in Table 1) also resulted in an increased cleavage by HNE.

Table 2. Hydrolysis of D-DY, D-DR, and N-ER by PR3: Michaelis (K_m) and Specificity (k_{cat}/K_m) Constants Obtained Using a Michaelis−Menten or a Substrate Inhibition Kinetics Model

name	K_m (μM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
D-DY_{FRET}	35.1[b]/27.4[c]	615921[b]/651275[c]
D-DR_{FRET}[a]	33.7[b]/21.4[c]	548753[b]/649976[c]
N-ER_{FRET}[a]	16.5[b]/11.5[c]	99848[b]/120047[c]

[a]No hydrolysis by HNE under the same experimental conditions. [b]Substrate inhibition model. [c]Michaelis−Menten kinetics model.

Determination of K_m and Specificity Constants for D-DY_{FRET}, D-DR_{FRET}, and N-ER_{FRET}. The specificity (k_{cat}/K_m) and Michaelis (K_m) constants were determined for D-DY_{FRET} and the two substrates identified as specific for PR3 from the percentages of cleavage analyzed by HPLC. We used two kinetic models, either substrate inhibition or Michaelis Menten (cf. Supporting Information Figure S4). The results are reported in Table 2. The specificity constants of these two FRET substrates are comparable, while N-ER_{FRET} yields the lowest K_m and specificity constant of the three substrates. The low k_{cat}/K_m measured for this substrate indicates a k_{cat} significantly lower than for the other two substrates. Among the three substrates N-ER_{FRET} is the one with the highest affinity for the enzyme. Working at lower substrate concentrations than for the determination of the percentages of hydrolysis, we do not observe any hydrolysis of D-DR_{FRET} and N-ER_{FRET} by HNE. We found k_{cat}/K_m values that are about 1 order of magnitude lower for D-DY_{FRET} and D-DR_{FRET} than previously reported values (7 000 000 and 3 400 000 $M^{-1}\cdot s^{-1}$, respectively), using lower substrate concentrations (<1 μM) than in the present study (2−256 μM).[5] This discrepancy is possibly due to the differences in the enzyme concentrations in the earlier experimental setup and the assumptions used for k_{cat}/K_m determination.

CONCLUSION

Molecular dynamics simulations of the substrate−enzyme complexes and subsequent free energy decompositions led us to suspect that EDDnp and Abz favorably interact, via van der Waals interactions, with PR3 and HNE. Using enzymatic assays, we show that N- and C-terminal FRET groups significantly contribute to enzymatic hydrolysis. We would therefore recommend a cautious approach while using Abz/EDDnp substrates in fluorescence assays and especially so for the determination of kinetic constants, as these might be biased by the low substrate concentrations used to avoid inner-filter effect. On the other hand and because of their improved interaction with PR3, the introduction of Abz and EDDnp groups in inhibitors of PR3 for in vitro diagnostic tools is an advantage over simpler substrates.[2] Other FRET groups containing aromatic rings will have the same ability to mediate van der Waals interactions and should yield the same effect. This study also reports a FRET substrate (N-ER_{FRET}) with high affinity for PR3, not cleaved by HNE, and that can form the basis for the development of peptidomimetic inhibitors. Moreover, the results of this study are relevant for the development of novel FRET substrates for the detection of NSPs in diagnoses of chronic inflammatory diseases.[2,9] Finally this study illustrates how MD simulations of enzyme/substrates

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Michaelis complexes followed by MM/PBSA free energy decompositions can qualitatively guide substrate design and evaluation of the energetic cost of replacing key charged amino acids by noncharged groups.

EXPERIMENTAL SECTION

Synthesis of Substrates. Rink amide MBHA (100−200 mesh, typically 0.400 g, 0.590 mmol/g loading) was swelled in a peptide synthesis vessel using 3 times bed volume of DMF and gentle agitation for ∼30 min. The solvent was removed by applying vacuum suction, and a solution of 20% piperidine in DMF was added covering the resin, followed by agitation for 5 min and draining by vacuum suction. This procedure was repeated twice more, and the deprotected Rink amide was washed by adding 3 times bed volume of DMF and agitating for 1 min and draining of the solvent. The washing procedure was repeated five times. Simultaneous with the washing, a solution of Fmoc-Gln-OH or Fmoc-Glu-EDDnp (4 equiv) and HBTU (3.9 equiv) in a minimum amount of DMF was prepared (Fmoc-Glu-EDDnp was prepared by modification of literature procedures,[10] and full experimental details are in the Supporting Information). To this solution was added DIPEA (8 equiv) followed by throughout mixing and immediate addition to the resin. The reaction mixture was agitated for 1 h after which the solvent was drained and the resin was washed five times with DMF and treated with 20% piperidine in DMF as described above. Subsequent coupling of the remaining amino acids using 4 equiv of the Fmoc-protected amino acid, 3.9 equiv of HBTU, and 8 equiv of DIPEA was performed following the same procedure. The deprotection of Fmoc-Asp(O*t*Bu)-OH and Fmoc-Asn(Trt)-OH and all subsequent Fmoc deprotections were carried out using a 20% piperidine in DMF solution containing 0.1 M HOBt in order to suppress aspartimide formation.[11] After the coupling and deprotection of the last amino acid/fluorophore the resin was dried-down by washing five times with DMF, CH_2Cl_2, MeOH, and hexane, respectively before it was dried by vacuum suction for ∼30 min. The dry resin was treated in the peptide synthesis vessel with 2 times bed volume of a mixture of TFA, TIS, and water (95:2.5:2.5, v/v) under gentle agitation for 3 h. The TFA mixture was drained off, and the resin was washed three times with fresh portions of TFA. The combined TFA solution was concentrated by rotatory evaporation, and the residue was precipitated by the addition of cold diethyl ether. The ether was removed and the residue triturated twice more with cold diethyl ether. The crude peptides were purified by reverse phase HPLC, and the combined fractions were lyophilized to give the pure peptides as fluffy white materials. Peptides containing the Abz and EDDnp groups were isolated as fluffy yellow materials. All substrates were found to be of >95% purity (HPLC 220 nm).

Proteolytic Analysis Using HPLC. The enzymes PR3 (EC 3.4.21.76) and HNE were purchased from Athens Research & Technology, Inc.. Purity was checked using SDS−PAGE gel, and PR3 was titrated with α1-proteinase inhibitor. Their activity was verified by analyzing the hydrolysis of Boc-Ala-Pro-nVal *p*-chlorothiobenzyl ester, in which cleavage was detected using 5,5-dithiobis(2-nitrobenzoic acid) and measuring absorbance at 412 nm. Lyophilized substrates were dissolved in 30% v/v DMF. The stock solution was further diluted with 50 mM Hepes, pH 7.4, 750 mM NaCl, supplemented with 0.05% (v/v) Igepal CA-630. For the proteolytic analysis, 90 μM substrate was incubated with 100 nM PR3 or HNE in 60 μL final reaction volume. Briefly, after a 30 min incubation at 37 °C the reaction was stopped by adding 5 μL of 10% trifluoroacetic acid (TFA) and incubating on ice for 10 min. This was followed by centrifugation for 10 min at 13000*g* at 4 °C. The supernatant was then transferred to HPLC vials. Hydrolysis products were separated and analyzed by reverse-phase HPLC using Shimadzu Prominence module HPLC instrument fitted with a Machery-Nagel C18 HD column. Samples were eluted using different gradients of water/acetonitrile/TFA (0.01% TFA) mobile phase for 55 min. Percentages of hydrolysis were calculated from relative areas under the curve of the (uncleaved) substrate peak. Each experiment was conducted three times with three different enzyme and substrate preparations.

K_m and k_{cat}/K_m Determination. An experimental setup similar to the one described above was used to determine Michaelis constants (K_m) for the fluorogenic substrates. We used eight different substrate concentrations ranging from 2 to 256 μM, while the final PR3 concentration was 0.4 nM. Aliquots were taken for analysis immediately after addition of the enzyme (t = 0) and every 6 min until t = 42 min. The product formation was determined by HPLC for each concentration and time point. This was then used to determine the reaction velocity and further K_m values using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA). The reduction in reaction velocity at high substrate concentrations led us to evaluate the Michaelis constants by fitting to a model of substrate inhibition.

Molecular Dynamics Simulations. We built the enzyme−peptide complexes using X-ray structures of PR3 and HNE.[6] The fluorogenic groups were added using the program Autodock 4.2.[12] Enzymes/substrate complexes were submitted to energy minimization followed by MD simulations with NAMD2.9[13] with the Charmm27 force field.[14] Sets of Charmm force field parameters for Abz and EDDnp were generated and validated as described in the Supporting Information. The simulations were performed in the NPT ensemble at a temperature of 300 K, with an integration time step of 1 fs. These consisted of four successive heating phases (10, 100, 200, and 300 K), a 150 ps equilibration phase, and a production phase of 2.5 ns. The last 2 ns of five production runs (five replicas with different initial velocities) are used for analysis. The protocol used to obtain the energetic contribution of all amino acids to the formation of the complexes is based on the MM/PBSA approach[15] (described in Supporting Information).

ASSOCIATED CONTENT

Supporting Information

MM/PBSA decompositions, enzyme kinetics, LC−MS results, and synthesis procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Abz, 2-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)-ethylenediamine; PR3, proteinase 3; HNE, human neutrophil elastase; MD, molecular dynamics

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