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Simple phosphonic inhibitors of human neutrophil elastase

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ABSTRACT

Herein, we describe the synthesis and resulting activity of a complex series of α-aminophosphonate diaryl esters as irreversible human neutrophil elastase inhibitors and their selectivity preference for human neutrophil elastase over several other serine proteases such as porcine pancreatic elastase, trypsin, and chymotrypsin. We synthesized and examined the inhibitory potency of several new simple Cbz-protected α-aminoalkylphosphonate diaryl esters that yielded several new HNE inhibitors, where one of the obtained compounds Cbz-Val^{P}(OC$_6$H$_4$-4-COOMe)$_2$ displayed an apparent second-order inhibition value at 33,015 M^{-1} s^{-1}.

Human neutrophil elastase (HNE), a serine protease classified within the chymotrypsin-like superfamily, which is secreted by azurophilic granules, was first described by Janoff and Scherer in 1968.[1] HNE has the ability to cleave almost every protein contained within the extracellular matrix (ECM) including, but not limited to, elastin, collagen, fibronectin, laminin, and proteoglycans.[1–5] The natural endogenous inhibitors for HNE are serpins such as α_1-PI, SLPI or α_2-macroglobulin.[6] Inactivation of serpins by the 'oxidative burst' of neutrophils affects the balance between inhibitors and HNE. Uncontrolled activity of HNE can lead to pathophysiological states such as chronic obstructive pulmonary disease (COPD), adult respiratory syndrome, pulmonary emphysema, cystic fibrosis or chronic bronchitis.[7–9] In addition, overexpression of elastase allows cancer cells within a tumor to develop and metastasize directly through degradation of ECM.[10]

Although numerous, structurally diverse inhibitors of human neutrophil elastase have been reported, none of them has demonstrated success in a clinical trial. One explanation for this failure could be the reversible nature of action of some of the inhibitors. Reversible inhibitors are not able to suppress the imbalance between natural endogenous inhibitors and elastase as incubation of elastase with both—reversible and irreversible inhibitors resulted in the formation of only covalent complexes (elastase-α_1-PI) and free, unbound reversible inhibitor molecules.

We also hypothesize that potent alkylating/acylating inhibitors of elastase could react even with some weak nucleophiles present in non-enzymatic proteins (like serum albumin). Such modified proteins could in turn act as antigens inducing an immune response. Thus, it seems that only irreversible and stable inhibitors such as α-aminoalkylphosphonates have the potential to overcome the limitations of current elastase inhibitors.[11]

The major advantage of the α-aminoalkylphosphonate diaryl esters is their potency, selectivity, and irreversible nature of action. These low molecular weight inhibitors of serine proteases are devoid of reactivity with cysteine, aspartyl or threonine proteases. The first described phosphonic-type inhibitors of elastase displayed relatively low potency and selectivity of action. However, elongation of the peptide chain resulted in higher potency of action as well as increased selectivity within the protease clan. The peptidyl derivative of a phosphonic valine analogue with a similar potency inhibited human neutrophil and porcine pancreatic elastases (MeO-Suc-Ala-Ala-Pro-Val^{P}(OPh)$_2$, k_{obs}/I = 7100 M^{-1} s^{-1}). Further studies led to the development of a more potent and a threefold more selective HNE inhibitor (Boc-Val-Pro-Val^{P}(OPh)$_2$, k_{obs}/I = 27,000 M^{-1} s^{-1}).[11] Although substitution of the phenyl ester ring with a chlorine atom at the *para* position and removal of the *t*-Boc protective group resulted in a twofold decrease in potency, however, the obtained inhibitor was approximately 26 times more active against human neutrophil elastase than elastase from porcine pancreas.[12] The high impact of the ester ring structures on the inhibitory potency of α-aminoalkylphosphonate diaryl esters has been demonstrated for other serine proteases such as trypsin,

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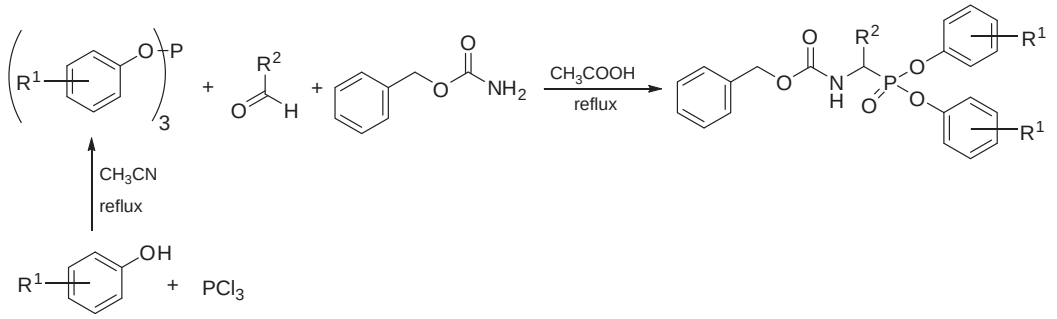
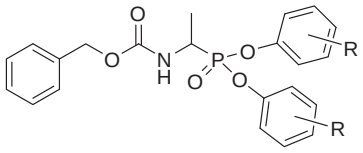
urokinase, cathepsin G or DPPIV.[13–15] Thus, we further investigated the influence of ester structure on the inhibitory activity of phosphonic-type inhibitors of human neutrophil elastase.

We synthesized several simple, Cbz-protected phosphonic analogues of aliphatic amino acids—alanine, valine, leucine, norvaline, norleucine, and amino butyric acid with different aromatic ester ring structures (Scheme 1). The inhibitory potency of the synthesized compounds against human neutrophil elastase as well as against other serine proteases such as porcine pancreatic elastase, chymotrypsin, and trypsin was measured using fluorescent



Scheme 1. Schematic representation of the synthesis of Cbz-protected phosphonic analogues of amino acids, where R^1 represents a phenyl ring substituent as presented in Tables 1–6 and R^2 represents methyl, ethyl, *iso*-propyl, *n*-propyl, *n*-butyl or *iso*-butyl chain.

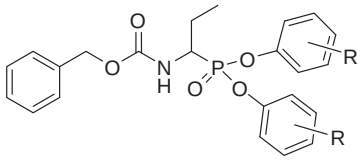
Table 1
Inhibition properties of phosphonic analogues of alanine (Cbz-Ala^P(OAr)$_2$) against HNE, PPE, chymotrypsin, and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
1	H	<50	NI	NI	NI
2	4-Ethyl	<50	NI	NI	NI
3	4-Isopropyl	<50	NI	NI	NI
4	4-*t*-Butyl	<50	<50	NI	NI
5	4-*S*-Methyl	<50	NI	NI	NI
6	4-Carbomethoxy	7722 ± 374	197 ± 39	1436 ± 56	1527 ± 5

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.

Table 2
Inhibition properties of Cbz-Abu^P(OAr)$_2$ toward HNE, PPE, chymotrypsin, and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
7	H	<50	NI	<50	NI
8	4-Methyl	NI	NI	<50	NI
9	2,5-Dimethyl	NI	NI	NI	NI
10	3,4-Dimethyl	NI	NI	<50	NI
11	4-Ethyl	<50	NI	<50	NI
12	4-Isopropyl	<50	<50	NI	NI
13	4-*t*-Butyl	NI	NI	<50	NI
14	4-*S*-Methyl	201 ± 6	<50	<50	NI
15	4-*O*-Methyl	<50	<50	<50	NI
16	4-Carbomethoxy	22,031 ± 932	1556 ± 27	1075 ± 35	1071 ± 44
17	4-Chloro	76 ± 5	<50	<50	<50
18	3-Chloro	346 ± 48	<50	<50	NI

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.

substrates. The influence of selected compounds on the proliferation of several cancer cell lines using Sivelestat (ONO-5046) as the reference compound was estimated.[16,17] A detailed description of the enzymatic and cell methods as well as the synthetic protocols can be found in the Supplementary data associated with this article.

Among all synthesized phosphonic analogues of alanine the highest potency of action was observed for a derivative containing a carbomethoxy substituent at the *para* position of the phenyl ester ring (**6**, k_2/K_i = 7722 M^{-1} s^{-1}). A 4-carbomethoxy substituent was only effective for the Ala^P derivatives displaying poor selectivity of action against other tested serine proteases (40 times more active against HNE than PPE, approximately 5 times less active against trypsin and chymotrypsin). Other alanine analogues (**1**–**5**) displayed no activity in the tested concentration range.

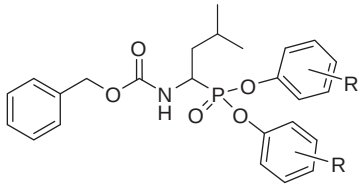
A much higher selectivity of action was observed for an Abu^P derivative with 4-carbomethoxy substitution within the phenyl ester ring (**16**, k_2/K_i = 22,031 M^{-1} s^{-1}) which displayed approximately 22 times more activity against HNE than against chymotrypsin and trypsin, but only 14 times more reactivity with PPE. Interestingly, a slight increase in activity against neutrophil elastase was also noticed for analogues with 3-Cl-phenyl (**18**, k_2/K_i = 346 M^{-1} s^{-1}), 4-*S*-methylphenyl (**14**, k_2/K_i = 201 M^{-1} s^{-1}), and 4-Cl-phenyl (**17**, k_2/K_i = 76 M^{-1} s^{-1}) ester rings when compared with their unsubstituted derivative **7**.

Out of the total synthesized phosphonic analogues of valine one compound, similar to analogues of Ala^P and Abu^P, contained a carbomethoxy substituent at the *para* position of the phenyl ester ring (**26**, k_2/K_i = 33,015 M^{-1} s^{-1}) and was the most potent inhibitor observed in this study. Compound **26** displayed almost absolute selectivity of action against the tested proteases (k_2/K_i <50 M^{-1} s^{-1} toward chymotrypsin and trypsin). Such results are somewhat unique for simple Cbz-protected α-aminoalkylphosphonate diaryl esters which usually inhibit a broad spectrum of serine proteases or display no activity at all. Interestingly, a valine analogue with a 4-methylthiophenyl ester group (**24**) displayed no inhibitory activity towards the tested proteases. In contrast, its oxidized homologue (**27**) with a 4-SO_2-methyl substituent was active against HNE (k_2/K_i = 15,945 M^{-1} s^{-1}) but its selectivity of action was rather poor, it displayed a similar potency against chymotrypsin (k_2/K_i = 12,660 M^{-1} s^{-1}), as well as lower activity against trypsin (k_2/K_i = 707 M^{-1} s^{-1}) and PPE (k_2/K_i = 288 M^{-1} s^{-1}).

Phosphonic analogues of leucine obtained in the presented studies lack activity against human neutrophil elastase. They are, however, potent inhibitors of chymotrypsin. The highest potency against HNE was observed for derivative **43**, which contained 4-carbomethoxyphenyl ester rings in its structure (**k_2/K_i** = 375 M^{-1} s^{-1}).

Similarly, among the synthesized analogues of *n*Val and *n*Leu, only derivatives with 4-carbomethoxy groups displayed the highest potency of action against HNE. Compound **54** was 6 times more

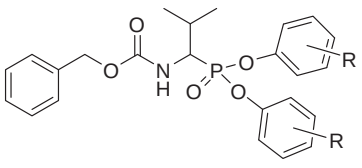
Table 4
Inhibition properties of Cbz-Leu^P(OAr)$_2$ toward HNE, PPE, chymotrypsin, and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
29	H	<50	<50	650 ± 32[a]	NI
30	4-Methyl	<50	<50	914 ± 228[a]	NI
31	2-Methyl	<50	NI	293 ± 102[a]	NI
32	2,5-Dimethyl	NI	<50	<50[a]	NI
33	3,4-Dimethyl	<50	<50	306 ± 3[a]	NI
34	2,3-Dimethyl	<50	<50	3500 ± 455[a]	NI
35	4-Ethyl	<50	<50	173 ± 21[a]	NI
36	4-Isopropyl	<50	<50	<50[a]	NI
37	4-*t*-Butyl	<50	<50	<50[a]	NI
38	2,3,5-Trimethyl	<50	<50	213 ± 70[a]	NI
39	3,4,5-Trimethyl	<50	<50	4425 ± 575[a]	NI
40	4-*S*-Methyl	<50	NI	18,300 ± 82[a]	NI
41	4-*O*-Methyl	NI	<50	<50	NI
42	3-*O*-Methyl	<50	<50	8760 ± 1570[a]	NI
43	4-Carbomethoxy	375 ± 13	NI	61,770 ± 5860[a]	<50
44	3-Carbomethoxy	<50	<50	3721 ± 418	NI
45	4-Chloro	<50	NI	2170 ± 390[a]	NI
46	3-Chloro	<50	<50	4430 ± 310[a]	NI

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.
[a] Data previously reported.[18]

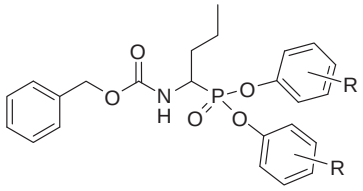
Table 3
Inhibition properties of Cbz-Val^P(OAr)$_2$ toward HNE, PPE, chymotrypsin, and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
19	H	NI	NI	NI	NI
20	2,5-Dimethyl	NI	NI	NI	NI
21	4-Ethyl	<50	<50	NI	NI
22	4-Isopropyl	NI	NI	NI	NI
23	4-(1,1,3,3-Tetramethyl)butyl	NI	NI	NI	NI
24	4-*S*-Methyl	<50	NI	<50	NI
25	4-*O*-Methyl	<50	<50	NI	NI
26	4-Carbomethoxy	33,015 ± 1445	NI	<50	<50
27	4-SO_2-methyl	15,945 ± 285	288 ± 31	12,660 ± 2643	707 ± 34
28	4-*t*-Butyl	<50	NI	NI	NI

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.

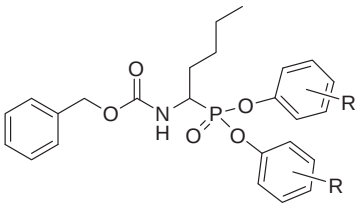
Table 5
Inhibition properties of Cbz-*n*Val^{P}(OAr)$_2$ toward HNE, PPE, chymotrypsin and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
47	H	<50	<50	<50	NI
48	2,3-Dimethyl	NI	NI	<50	NI
49	4-Ethyl	<50	<50	<50	NI
50	2,3,5-Trimethyl	NI	NI	<50	NI
51	3,4,5-Trimethyl	NI	NI	NI	NI
52	4-*S*-Methyl	<50	<50	<50	NI
53	4-*O*-Methyl	NI	NI	<50	NI
54	4-Carbomethoxy	17,858 ± 418	2868 ± 88	3040 ± 70	<50

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.

Table 6
Inhibition properties of Cbz-*n*Leu^{P}(OAr)$_2$ toward HNE, PPE, chymotrypsin, and trypsin



Compound	R	k_2/K_i [M^{-1} s^{-1}]			
		HNE	PPE	Chymotrypsin	Trypsin
55	H	<50	NI	NI	NI
56	4-*S*-Methyl	NI	NI	<50	NI
57	4-*O*-Methyl	<50	<50	<50	NI
58	4-Carbomethoxy	452 ± 26	<50	338 ± 14	<50
59	4-Isopropyl	<50	<50	<50	NI
60	4-*t*-Butyl	<50	<50	NI	NI
61	4-Chloro	<50	<50	<50	NI

NI—less than 5% of inhibition was observed after incubation of enzyme with the inhibitor (5 μM) at 37 °C.

active against HNE (k_2/K_i = 17,858 M^{-1} s^{-1}) than chymotrypsin and PPE with only a slight inhibition of trypsin (k_2/K_i <50 M^{-1} s^{-1}). Cbz-*n*Leu^{P}(O-4-C_6H_4–$COOCH_3$)$_2$ (**58**) was the only active analogue of this group against HNE (k_2/K_i = 452 M^{-1} s^{-1}) but also inhibited chymotrypsin with similar potency.

The presented data clearly suggest that the highest activity of simple Cbz-protected phosphonic analogues of amino acids is the result of the 4-carbomethoxy substituent within the aromatic ester ring. The strong electrowithdrawing properties of this group might lead to the increased inhibition of the target enzyme. It seems likely that the effect of this substituent results in high electrophilic potential of the phosphorus atom that facilitates the enzyme's nucleophile attack which directs the inhibition pattern. The interaction between the S1 pocket and the P1 side chain seems to be less important for a particular group of phosphonic amino acid derivatives as the observed inhibitory effect is mostly the result of the nature of the phenyl ester ring substituents. When comparing the activity of derivatives with the same ester structure the highest activity was observed for analogues with the *iso*-propyl and ethyl chains as the P1 group. Shorter or longer chains resulted in decreased potency of action.

Table 7
An anti-proliferative effect of selected compounds on human lung adenocarcinoma cell line (A549), human breast cancer cell line (MCF-7), human colon carcinoma cell line (LoVo), and human multidrug-resistant colon carcinoma cell line (LoVoDX)

Compound	A549	Lovo	LovoDX	MCF7
Sivelestat	0	20% ± 2[a]	15% ± 3[a]	61% ± 5[a]
6	25% ± 7[a]	47 μg/ml ± 15	46% ± 4[a]	40 μg/ml ± 6
16	31% ± 2[a]	73 μg/ml ± 21	38% ± 2[a]	49% ± 3[a]
26	46% ± 3[a]	32 μg/ml ± 6	54% ± 10[a]	47 μg/ml ± 6
27	5% ± 5[a]	15% ± 6[a]	0	19% ± 5[a]
43	3% ± 3[a]	22% ± 6[a]	6% ± 6[a]	8% ± 6[a]
54	40% ± 6[a]	35 μg/ml ± 4	47% ± 15[a]	52 μg/ml ± 7

[a] IC_{50} value could not be calculated, inactive in the investigated range, results shown as percentage of cell growth inhibition in a concentration 100 μg/ml.

The influence of selected inhibitors on the proliferation of cancer cell lines showed that the most active was compound **26**, which also displayed the highest inhibition of human neutrophil elastase in the enzyme kinetic assay. It decreased the growth of LoVo and MCF7 cell lines by 50% at 32 and 47 μg/ml, respectively. Slightly less inhibition was observed when LoVoDX and A549 cell lines were treated with **26**. The effect of cell growth inhibition observed for compound **54** was slightly lower when compared to compound **26**, but it displayed almost 2 times lower inhibition of HNE. Similar behavior was observed for compound **6**. For all three analogues (**6**, **26**, and **54**) the growth inhibition effect was much higher than the effect observed for the control compound Sivelestat, which in our studies displayed almost no activity towards the tested cell lines. Importantly, decreasing the potency of action of HNE inhibitors as well as their selectivity, results in less inhibition of cancer cell line growth (Table 7).

The presented work describes the effect of the phenyl ester group substituents of simple Cbz-protected phosphonic analogues of amino acids on the activity of human neutrophil elastase. It also provides insight into further development of more potent and selective inhibitors of this class of compounds.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.083.

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