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Phosphonic pseudopeptides as human neutrophil elastase inhibitors—a combinatorial approach

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ABSTRACT

Here we present a simple and rapid method for the construction of phosphonic peptide mimetic inhibitor libraries—products of Ugi and Passerini multicomponent condensations—leading to the selection of new biologically active phosphonic pseudopeptides. As the starting isonitriles, 1-isocyanoalkylphosphonate diaryl ester derivatives were applied. The structure of the synthesized inhibitors was designed to target human neutrophil elastase, a serine protease whose uncontrolled activity may lead to development of several pathophysiological states such as rheumatoid arthritis, cystic fibrosis or tumor growth and invasion. After screening the inhibitory activity of our constructed libraries, the most active compounds were synthesized as single molecules. One of the obtained inhibitors, Cbz-Met-O-Met-Val^{P}(OC_6H_4-*p*-Cl)$_2$, displayed apparent second-order inhibition value at 40,105 M^{-1} s^{-1} as the diastereomers mixture. Inhibition potency and selectivity of action toward other serine proteases as well as the results of initial in vitro experiments regarding inhibitors influence on cancer cell proliferation are presented.

1. Introduction

Upon inflammatory stimulation leukocytes release three major serine proteases: cathepsin G (catG), proteinase 3 (P-3), and neutrophil elastase (HNE).[1] A broad spectrum of substrate specificity and potency of action places elastase among most destructive proteases in humans.[2] The proteolytic activity of HNE in the healthy body is precisely controlled by natural, endogenous inhibitors belonging to the serpin superfamily. The most important serpins which function as HNE inhibitors are α-1 proteinase inhibitor (α-1 PI) and α_2-macroglobulin.[3] Disruption of the imbalance between elastase and its inhibitors may lead to development of diseases such as chronic obstructive pulmonary disease (COPD), pulmonary emphysema or cystic fibrosis.[4] Uncontrolled activity of HNE may also participate in cancer cell growth and metastasis.[5] The process of extracellular matrix degradation by HNE can occur directly through proteolysis of structural components such as elastin or collagens, or indirectly *via* zymogen activation of the plasminogen–plasmin system.[6]

Due to the structural analogy of α-aminoalkylphosphonate diphenyl esters to the transition state observed during the peptide bond cleavage catalyzed by proteases, this class of compounds displays interesting inhibitory properties.[7] Aromatic esters of α-aminoalkylphosphonates are potent, irreversible inhibitors which react exclusively with the catalytic hydroxyl group of serine proteases. Good stability and the lack of reactivity with other classes of proteases such as cysteine, aspartyl, threonine or metalloproteinases have increased investigations into this class of inhibitors.[8] Moreover, simple modification of the phosphonic analogue side chain or synthesis of their peptidyl derivatives allows for the design of specificity between even highly similar proteases.[9]

Currently, numerous, structurally diverse α-aminoalkylphosphonate diphenyl esters have been synthesized and their inhibitory properties were examined against target proteases including trypsin,[10] granzymes,[11] uPA[12–14] or DPPIV.[15] They have also been used for the design of activity-based probes able to detect enzymatically active serine proteases in cell cultures.[16,17]

However, despite the passage of more than 30 years between the publication of the first paper concerning the synthesis of α-aminoalkylphosphonate diphenyl esters[18] no attention has been paid to the construction of inhibitor libraries based on these compounds. Unnatural peptidyl derivatives like depsipeptides or peptides with N-substituted amides may display improved stability in plasma since they are less susceptible to degradation by enzymes.

Here we present a simple and effective method for the construction of α-aminoalkylphosphonate diphenyl ester-based libraries. Described methodology and the results presented below represent an example of this technology application. In light of our previous

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research on the synthesis of 1-isocyanoalkylphosphonates we applied two known multicomponent reactions—Passerini-type three component condensation and Ugi-type four component reaction (Fig. 1). The great potential and structural variety of final products which results from both reactions are limited only by the researcher's imagination. In the case of Ugi multicomponent condensation the combination of 40 different carboxylic acids, 40 different aldehydes, 40 different amines, and 40 different isonitriles leads to the potential synthesis of 2,560,000 different compounds.[19–21]

In both Passerini and Ugi reactions we used 1-isocyanoalkylphosphonates as the starting substrates. We included phosphonic analogues of amino acids substituted at the phenyl ester rings in the construction of the libraries as these have been shown to increase the potency of inhibitor action.[22] We focused on the design of libraries which target human neutrophil elastase. The selectivity of inhibitors action was evaluated using chymotrypsin and trypsin. The data obtained from the first experiments on the influence of the most active inhibitors on cancer cell proliferation will also be presented.

2. Results and discussion

Using *N*-Cbz-protected α-aminoalkylphosphonate diaryl esters as the starting compounds we synthesized several isocyanide derivatives which were subsequently used in the multicomponent reactions of both types. The isocyanides used in the presented studies were phosphonic analogues of Val, Ala, and Abu. The amines and the carbonyl components which were used for the inhibitor libraries construction were selected to obtain high structural diversity of target molecules (aliphatic, aromatic, and bulky groups). That allowed us to determine the applicability and limitation of the method itself. Also an introduction of structurally diversified groups could led to discovery of possibly new recognition sites between target enzyme and the inhibitor molecule which could guide us for future development of elastase (as well as other serine proteases) inhibitors.

The libraries were constructed in order to obtain four desired compounds in one reaction mixture. One library contained 24 reaction mixtures with 4 products in each mixture (the total number was 96 compounds per library). Firstly, two mixtures composed of four different aldehydes in methanol were prepared. For the Passerini reaction 1 equiv of a carboxylic acid substrate methanol solution was added followed by the addition of 1 equiv of the aldehyde mixture (0.25 equiv of each aldehyde). The solution was allowed to react for 10 min, then 1 equiv of 1-isocyanoalkylphosphonic diaryl ester was added. In the case of the Ugi multicomponent condensation, 1 equiv of amine component was allowed to react with the mixture of aldehydes for 2 h at room temperature. Next, the carboxylic acid component was added followed by the addition of isocyanide derivative of phosphonic acid diaryl ester. Both Passerini and Ugi reactions were performed at room temperature for 3 days. Each reaction was then quenched by the addition of 1 equiv of HCl methanol solution and evaporated to dryness. The residue was dissolved in dimethylsulfoxide in order to obtain a 10 mM concentration of each of the final products (assuming 100% reaction yield). These mixtures were next screened for inhibitory activity toward human neutrophil elastase using the incubation method. All the libraries were subsequently diluted to select the most active compounds. The starting isocyanides were also examined and no inhibition was observed in any case.

From the prepared *heat-maps* we were able to select inhibitor mixtures with the highest potency of action. In the next step potential inhibitors were synthesized as pure compounds in the same manner. Their ability to inhibit the proteolytic activity of human neutrophil elastase as well as the selectivity of action towards other serine proteases was examined. The *heat-map* analysis showed high diversity of inhibitory activity among constructed libraries. The most active compound among all synthesized were phosphonic depsipeptides (Passerini products, Fig. 2). The Ugi products (Fig. 3) displayed lower inhibition properties as compared to the Passerini-based products. In both types of condensation products the highest inhibition was observed for derivatives of 1-isocyano-2-methylpropanephosphonic acid di(4-*S*-methylphenyl) ester (**1d**) although the Passerini-based library displayed higher potency. Interestingly, a library based on 1-isocyano-2-methylpropanephosphonic acid di(4-chlorophenyl) ester (**1f**) showed the highest potency among all synthesized mixtures. The higher activity of dichlorophenyl esters as compared to diphenyl esters may be the result of the electrowithdrawing properties of the chlorine atom facilitating release of the *p*-Cl-phenoxy group.

For the most active inhibitor mixtures single compounds were synthesized separately and their inhibitory properties were re-examined. Analysis of pure, single compounds showed that the most active derivative among all of the synthesized was Cbz-Met-O-Met-Val^{P}(O-C_6H_4-4-Cl)$_2$ (**P5**) which displayed k_{obs}/[I] value at 40,105 M^{-1} s^{-1} being absolutely selective against chymotrypsin and trypsin (Table 1). From the same group of inhibitors compound **P3** (Cbz-Met-O-Val-Val^{P}(O-C_6H_4-4-*Cl*)$_2$ showed good potency of action (k_{obs}/[I] value 20,500 M^{-1} s^{-1}). Even compound **P3** had no activity against trypsin, it inhibited chymotrypsin (15%) at 2.5 μM after 30-min incubation. Other compounds of this group displayed moderate or poor potency of action. Parallel derivatives (Table 2) which differed only at the *para* substituent (methylthio group

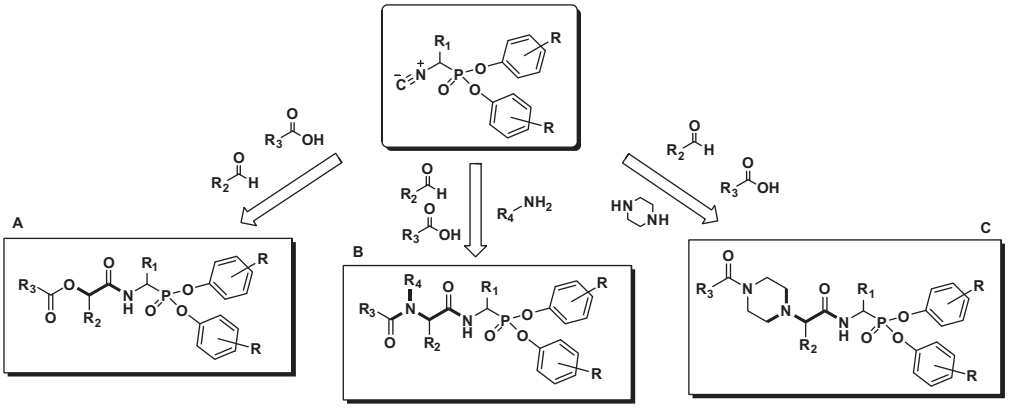


Figure 1. Schematic representation of the Passerini-type (A) and Ugi-type (B and C) multicomponent condensation used for construction of inhibitor libraries based on the of 1-isocyanoalkylphosphonate diaryl ester moiety.

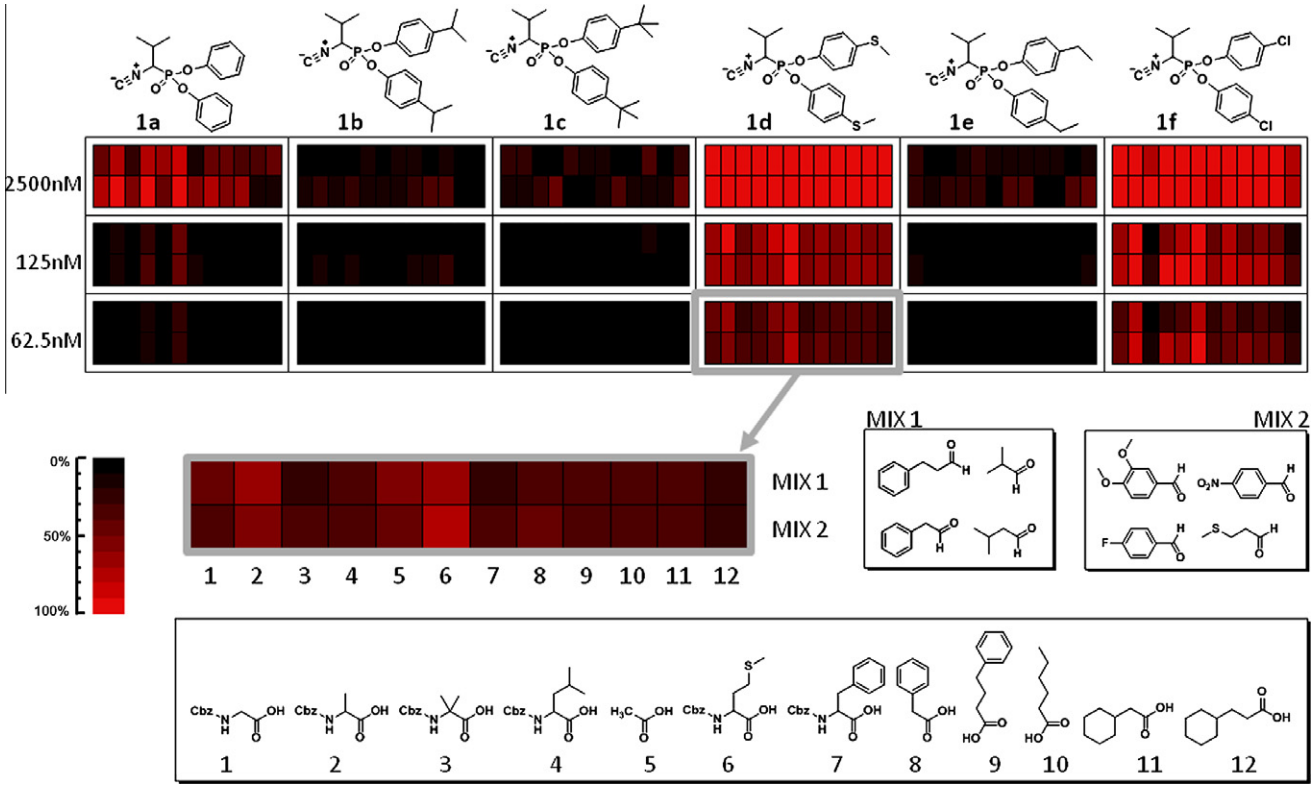


Figure 2. Schematic representation of heat-maps obtained for Passerini-based inhibitor libraries of phosphonic pseudopeptides. Each square represents a mixture of four products. MIX 1—equimolar mixture of four aldehydes (phenylpropionaldehyde, isobutylaldehyde, isovaleraldehyde, and phenylacetaldehyde), MIX 2—equimolar mixture of 4-nitrobenzaldehyde, 4-fluorobenzaldehyde, 3,4-dimethoxybenzaldehyde and 3-(methylthio)propionaldehyde. The percent of inhibition represents a ratio of enzyme activity after incubation with the inhibitors mixture into the control sample. Typical serial dilutions (2.5 μM, 0.125 μM, and 0.0625 μM) show decreased level of inhibitory activity which allowed for the selection of the most active compounds.

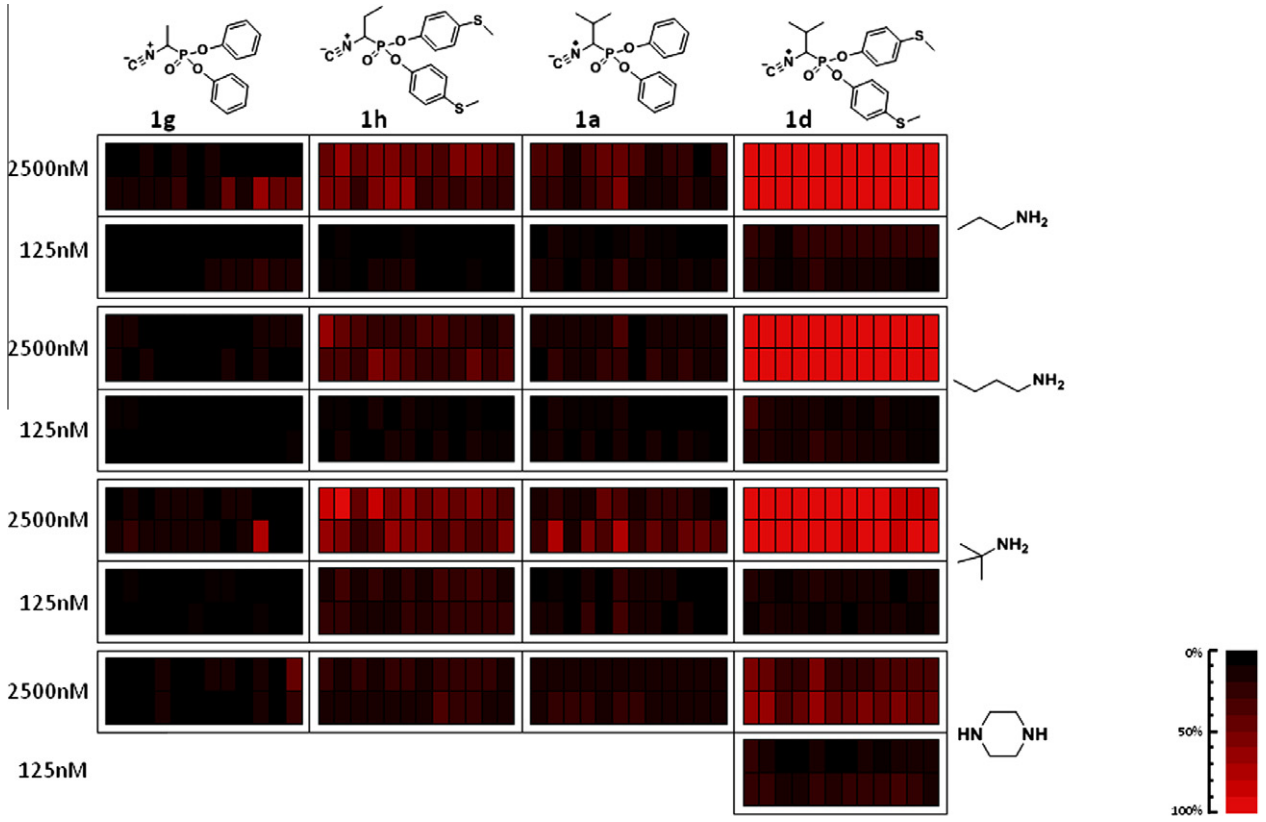
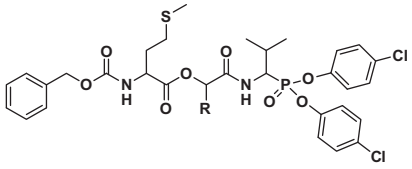


Figure 3. Schematic representation of heat-maps obtained for Ugi-based inhibitor libraries of phosphonic pseudopeptides. Details as described for Figure 2.

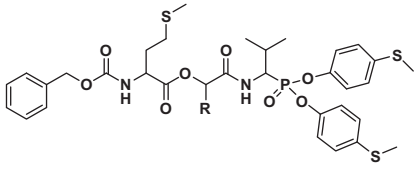
Table 1
Inhibitory activity of phosphonic peptide-mimetics obtained by the application of Passerini-type multicomponent condensation—derivatives of 1-isocyano-2-methylpropane-phosphonic acid di(4-chlorophenyl) ester



Compound	R	k_{obs}/[I] (M^{-1} s^{-1})		
		HNE	Chymotrypsin[a,b]	Trypsin[b]
P1	$-CH_2CH_2C_6H_5$ **(hPhe)**	1620	NI	NI
P2	$-CH_2CH(CH_3)_2$ **(Leu)**	7250	NI	NI
P3	$-CH(CH_3)_2$ **(Val)**	20,500	15%	NI
P4	$-CH_2C_6H_5$ **(Phe)**	695	NI	NI
P5	$-CH_2CH_2SCH_3$ **(Met)**	40,105	NI	NI
P6	$-C_6H_3(m,p\text{-}OCH_3)_2$ **(3,4-OMe)Phg**	165	21%	NI
P7	$-C_6H_4(p\text{-}NO_2)$ **(4-NO_2)Phg**	165	NI	NI
P8	$-C_6H_4(p\text{-}F)$ **(4-F)Phg**	215	16%	NI

[a] The Percent of enzyme inhibition after incubation for 30 min at 37 °C with 2.5 μM of inhibitor relative to the control sample.
[b] NI—no inhibition was observed after 30 min incubation of enzyme with the inhibitor (2.5 μM) at 37 °C.

Table 2
Inhibitory activity of phosphonic peptide-mimetics obtained by the application of Passerini-type multicomponent condensation—derivatives of 1-isocyano-2-methylpropane-phosphonic acid di(4-*S*-methylphenyl) ester



Compound	R	k_{obs}/[I] (M^{-1} s^{-1})		
		HNE	Chymotrypsin[a,b]	Trypsin[b]
P9	$-CH_2CH_2C_6H_5$ **(hPhe)**	765	NI	NI
P10	$-CH_2CH(CH_3)_2$ **(Leu)**	4400	NI	NI
P11	$-CH(CH_3)_2$ **(Val)**	5050	15%	NI
P12	$-CH_2C_6H_5$ **(Phe)**	605	NI	NI
P13	$-CH_2CH_2SCH_3$ **(Met)**	5900	14%	NI
P14	$-C_6H_3(m,p\text{-}OCH_3)_2$ **(3,4-OMe-Phg)**	3800	22%	NI
P15	$-C_6H_4(p\text{-}NO_2)$ **(4-NO_2-Phg)**	430	NI	NI
P16	$-C_6H_4(p\text{-}F)$ **(4-F-Phg)**	4850	NI	NI

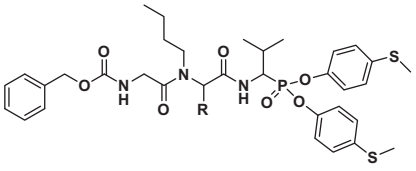
[a] The Percent of enzyme inhibition after incubation for 30 min at 37 °C with 2.5 μM of inhibitor relative to the control sample.
[b] NI—no inhibition was observed after 30 min incubation of enzyme with the inhibitor (2.5 μM) at 37 °C.

instead of chlorine atom) displayed lower inhibition values. Compound **P13** (Cbz-Met-O-Met-Val^P(O-C_6H_4-4-*S*-Me)$_2$ was almost sevenfold less reactive than compound **P5** (k_{obs}/[I] = 5900 M^{-1} s^{-1}), but it also inhibited chymotrypsin (14% of inhibition, at 2.5 μM after 30-min incubation). Similarly, derivative **P11** was approximately fourfold less potent than 4-chloro derivative **P3** (k_{obs}/[I] = 5050 M^{-1} s^{-1}) displaying similar activity toward chymotrypsin. Interestingly, an improvement of inhibitory potency was observed for compounds **P14** (k_{obs}/[I] = 3800 M^{-1} s^{-1}) and **P16** (k_{obs}/[I] = 4850 M^{-1} s^{-1}) when compared to their 4-chloro analogues **P6** and **P8** (k_{obs}/[I] values 165 M^{-1} s^{-1} and 215 M^{-1} s^{-1}, respectively).

The Ugi-derived products (**U1**–**U8**) displayed poor activity against tested serine proteases (Tables 3 and 4). The most potent among them were Cbz-Gly-(*N*-*n*-butyl)-hPhe-Val^P(OC_6H_4-*p*-*S*-Me)$_2$ (**U1**, k_{obs}/[I] = 1415 M^{-1} s^{-1}) and Cbz-Gly-(*N*-*n*-butyl)-Phe-Val^P(OC_6H_4-*p*-*S*-Me)$_2$ (**U4**, k_{obs}/[I] = 1415 M^{-1} s^{-1}) but they also inhibited chymotrypsin after incubation.

In general, the obtained compounds displayed lower inhibitory activity as compared to previously published highly potent

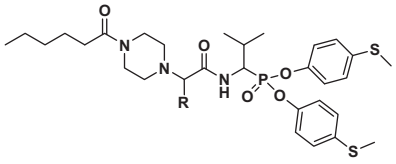
Table 3
Inhibitory activity of phosphonic peptide-mimetics obtained by the application of Ugi-type multicomponent condensation—derivatives of 1-isocyano-2-methylpropanephosphonic acid di(4-*S*-methylphenyl) ester



Compound	R	k_{obs}/[I] (M^{-1} s^{-1})		
		HNE	Chymotrypsin[a,b]	Trypsin[b]
U1	$-CH_2CH_2C_6H_5$ **(hPhe)**	1415	15%	NI
U2	$-CH_2CH(CH_3)_2$ **(Leu)**	145	NI	NI
U3	$-CH(CH_3)_2$ **(Val)**	495	8%	NI
U4	$-CH_2C_6H_5$ **(Phe)**	1360	14%	NI

[a] The Percent of enzyme inhibition after incubation for 30 min at 37 °C with 2.5 μM of inhibitor relative to the control sample.
[b] NI—no inhibition was observed after 30 min incubation of enzyme with the inhibitor (2.5 μM) at 37 °C.

Table 4
Inhibitory activity of phosphonic peptide-mimetics obtained by the application of modified Ugi-type multicomponent condensation—derivatives of 1-isocyano-2-methylpropanephosphonic acid di(4-*S*-methylphenyl) ester



Compound	R	k_{obs}/[I] (M^{-1} s^{-1})		
		HNE	Chymotrypsin[a]	Trypsin[a]
U5	$-CH_2CH_2SCH_3$ **(Met)**	140	250	NI
U6	$-C_6H_3(m,p\text{-}OCH_3)_2$ **(3,4-OMe-Phg)**	125	NI	NI
U7	$-C_6H_4(p\text{-}NO_2)$ **(4-NO_2-Phg)**	140	NI	NI
U8	$-C_6H_4(p\text{-}F)$ **(4-F-Phg)**	330	NI	NI

[a] NI—no inhibition was observed after 30 min incubation of enzyme with the inhibitor (2.5 μM) at 37 °C.

phosphonic inhibitors of human neutrophil elastase. Nevertheless, it is necessary to highlight that the Ugi and Passerini products obtained here are mixtures of diastereoisomers. Regardless of the fact that these are mixed compounds, our results place some of them among the most active HNE diarylphosphonate inhibitors. Additionally, a major advantage of this new class of inhibitors may be their increased stability due to the limited susceptibility by proteases.

Passerini products (**P2**, **P3**, **P5**, **P13**) and Ugi products (**U1**, **U2**, **U4**), which displayed high potency of human neutrophil elastase inhibition were selected for in vitro studies. The preliminary data regarding the influence of synthesized inhibitors on normal and cancer cell growth showed that the highest inhibitory effect was observed for Ugi-derived products. The treatment showed a dose-dependent effect. Compound **U1** showed the greatest effect on cancer cell (A549), where concentrations between 125 μM and 500 μM inhibited more than 50% of cell growth. Importantly, exposure of cells to 250 μM of **U1** was not toxic to normal fibroblasts whereas even 125 μM was toxic to cancer cells (Fig. 4). For all tested compounds the toxicity level for HGF-s cells was approximately 500 μM, however a much lower concentration was required to induce a toxic effect in A549 cancer cells. Treatment with compound **U1** at 250 μM decreased cell growth by 70% in A549 cells, and by 10% in HGF-s cells.

Passerini products, although displaying a toxic effect toward normal human fibroblasts at high concentrations showed moderate inhibition of cancer cells at 125 μM and 250 μM. Following treatment of cells with compound **P5** (the most potent human neutrophil elastase inhibitor obtained in presented research) at 500 μM decreased cell growth by 60%, and at 250 μM by 25% in A549 cells. Among all the tested Passerini condensation products the most active was compound **P2** which at 500 μM inhibited 75% of cancer cells growth (Fig. 4).

It is important to note that an increased growth of fibroblasts was observed after treatment with all tested compounds where no such effect was observed for the A549 cell line. These results could represent the compounds influence on matrix dehydrogenase expression level which activity is measured by the MTT assay.

Additionally, when the control compound 4-MeO-C_6H_4-CO-Val-Pro-Val^P(OC_6H_4-4-*S*-CH_3)$_2$ was used[8]—a classic phosphonic peptide derivative, potent inhibitor of human neutrophil elastase (k_{obs}/[I] = 207,000 M^{-1} s^{-1}) no toxic effect against A549 cells was observed. That may prove the advantage of phosphonic peptide-mimetics (Passerini or Ugi products) over classic peptidyl derivatives.

The methodology described here allows us to obtain and screen new phosphonic-based peptide-mimetics in great number within just a few days. We synthesized and screened more than 2100 new compounds which led to select new potent and selective inhibitors of human neutrophil elastase.

Our presented work describes an example of the application of phosphonic isocyanide-based combinatorial chemistry methods for inhibitor library construction. It also suggested the possibility of generating libraries other than Passerini or Ugi multicomponent condensation products by the application of phosphonic isonitriles. Moreover, just by simply changing the P1 side chain the construction of inhibitors toward other serine proteases could be generated. Our laboratory is currently investigating the library construction method optimization as well as its application for inhibitors of different serine proteases synthesis and our results will be published in due course.

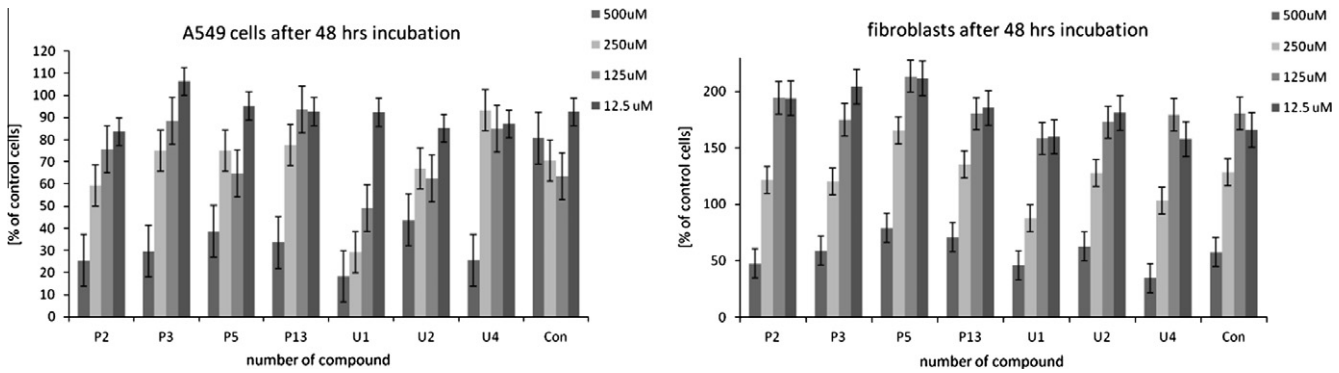


Figure 4. Lung cancer cell line A549 (A) and normal fibroblasts (B) growth inhibition by obtained HNE phosphonic peptide-mimetics. As the control compound 4-MeO-C_6H_4-CO-Val-Pro-Val^P(OC_6H_4-4-*S*-CH_3)$_2$ was applied.

3. Materials and methods

Carboxylic acids, phenols, triphenyl phosphite, phosphorus chloride, phosphorus oxychloride, and all solvents were purchased from Sigma–Aldrich. Aldehydes were purchased from Lancaster. Protected amino acids were purchased from IRIS Biotech GMBH.

3.1. Chemical synthesis

Starting phosphites (except commercially available triphenyl phosphite), Cbz-protected aminophosphonates, their *N*-formyl derivatives, and 1-isocyanoalkylphosphonates were prepared as described previously.[18,23]

3.1.1. General procedure for synthesis of substituted at the phenyl ester rings α-*N*-Cbz-protected aminophosphonates

In general, 0.03 mol of substituted phenol was dissolved in 30–40 ml of acetonitrile and 0.01 mol of phosphorus chloride was added. After refluxing the mixture for 4 h the volatile elements were evaporated and the crude phosphite was used in the next step. To the crude phosphite 0.01 mol of benzyl carbamate, 0.012 mol of an aldehyde, and 25 ml of glacial acetic acid were added. The reaction was performed at 80 °C for 2 h. After evaporation the resulting oily residue was dissolved in 100 ml of methanol and left for crystallization at −20 °C resulting in the desired α-*N*-Cbz-protected aminophosphonate aromatic esters in 30–60% yield. If necessary, re-crystallization was performed by dissolving the product in hot chloroform (5 ml), addition of 50 ml methanol and leaving at −20 °C.

3.1.2. General procedure for synthesis of α-*N*-formyl-aminophosphonate diaryl esters

First, the *N*-Cbz protective group was removed with 33% HBr in acetic acid solution—0.5 mmol of α-*N*-Cbz-aminophosphonate diaryl ester was dissolved in 2 ml of 33% HBr/AcOH. The reaction was performed at room temperature for 2 h. The volatile elements were evaporated and the resulting product was crystallized from a MeOH/diethyl ether system. The product was than filtered and air-dried. In the next step hydrobromide salt of 1-aminoalkylphosphonate diaryl ester (0.02 mol) was dissolved in methylene chloride (25 ml) in the presence of triethylamine (0.1 mol) and acetic-formic anhydride (0.03 mol) was added slowly at room temperature. The progress of the reaction was monitored by TLC. After the reaction was completed the mixture was evaporated, dissolved in ethyl acetate (the precipitating salt was filtered off), and washed with 5% $NaHCO_3$ aq and brine. After drying over Na_2SO_4 the solvent was evaporated and the resulting *N*-formyl derivative was used directly for the isocyanide synthesis (**F1**–**F8**, see Supplementary data).

3.1.3. 1-Isocyanoalkylphosphonate diaryl esters synthesis

α-*N*-Formyl-aminoalkylphosphonate diaryl ester (0.01 mmol) was dissolved in freshly distilled, dried over P_2O_5, dichloromethane (30–40 ml) and triethylamine (0.2 mmol) was added. The mixture was cooled to 0 °C and $POCl_3$ (0.04 mmol) was added dropwise. The progress of the reaction was monitored by TLC. After completion the reaction was quenched by slow addition of saturated aqueous solution of $NaHCO_3$. The mixture was extracted three times with $NaHCO_3$ and brine. After drying over Na_2SO_4 the solvent was evaporated. Resulting crude product was dissolved in minimal volume of chloroform and passed through the layer of silica gel to remove all polymeric impurities. TLC analysis showed a single spot. The solvent was evaporated and the oily product with characteristic isocyanate odor was obtained (**1a**–**1h**, see Supplementary data).

3.2. Inhibitor library synthesis

Synthesis of all libraries was performed in dry methanol. The final concentration of the reagents was kept as high as possible in order to increase the yield of the final products.

3.2.1. Synthesis of Passerini-based library

All substrates were dissolved in dry methanol to a 5 mM final concentration. In the reaction tubes 1 mmol of equimolar mixture (MIX I) of aldehydes was added into tubes labeled 1–12, and MIX II into tubes 13–24 (Fig. 2). To each tube 1 mmol of carboxylic compound methanol solution was added as follows: Cbz-Gly-OH (tubes 1 and 13), Cbz-Ala-OH (tubes 2 and 14), Cbz-Aib-OH (tubes 3 and 15), Cbz-Leu-OH (tubes 4 and 16), acetic acid (tubes 5 and 17), Cbz-Met-OH (tubes 6 and 18), Cbz-Phe-OH (tubes 7 and 19), phenylacetic acid (tubes 8 and 20), phenylbutyric acid (tubes 9 and 21), hexanoic acid (tubes 10 and 22), cyclohexylacetic acid (tubes 11 and 23), and cyclohexylpropionic acid (tubes 12 and 24). The mixtures were allowed to react at room temperature for 15 min and 1-isocyanoalkylphosphonate diaryl ester methanolic solution was added into all 24 reaction tubes. The mixtures were left at room temperature for 3 days. Into each of the tube 1 mmol of HCl in methanol was added to quench the reaction. The volatile components were evaporated and remaining residue was dissolved in dimethylsulfoxide (2.5 ml) to obtain 100 mM concentration of each target compound of the mixture. The solutions were kept at −86 °C prior to use in library enzymatic inhibition studies.

3.2.2. Synthesis of Ugi-based library

All the substrates were dissolved in methanol to the final concentration at 5 mM. Into all 24 reaction tubes 1 mmol of appropriate amine (Fig. 3) and 1 mmol of equimolar mixture of aldehydes (1–12 mixture I, 13–24 mixture II) were added. The reactions were left to react for 2 h at room temperature. After the carboxylic substrate solution (1 mmol) was added into each tube as described above. Next, a solution of 1-isocyanoalkylphosphonate diaryl ester was added into each tube (1 mmol) and the reaction was left for 3 days at room temperature. The reaction was quenched with HCl/MeOH (1 mmol) and evaporated to dryness. The mixtures were dissolved in DMSO (2.5 ml) and stored at −86 °C until the measurement of their inhibitory properties.

3.3. Single inhibitor synthesis

The single inhibitors were synthesized similarly to the method described above. The difference was that a single aldehyde was used instead of aldehyde mixture.

3.3.1. Synthesis of Passerini-based single product

An aldehyde (10 mmol) and a carboxylic acid (10 mmol) were dissolved in methanol. After 15 min 1-isocyanoalkylphosphonate diaryl ester was added into the reaction mixture. The solution was left at room temperature for 3 days with gentle stirring. After quenching the reaction with HCl/MeOH (10 mmol) volatile elements were evaporated and the residue was dissolved in ethyl acetate. The organic solution was washed with 5% $NaHCO_3$, 5% citric acid aqueous solution and finally with brine. After drying over $MgSO_4$ the solvent was evaporated and the product was purified by column chromatography on silica gel using ethyl acetate/chloroform mixture as the eluent (**P1**–**P16**, see Supplementary data).

3.3.2. Synthesis of Ugi-based single product

A mixture of an amine (10 mmol) and an aldehyde (10 mmol) was left to react for 2 h at room temperature. To this mixture carboxylic derivative (10 mmol) and 1-isocyanoalkylphosphonate diaryl ester were added, respectively. The reaction was performed at room temperature for 3 days with gentle stirring. The reaction was stopped by addition of HCl/MeOH solution, evaporated to dryness and re-dissolved in ethyl acetate. After extraction with 5% $NaHCO_3$, citric acid, and brine, the solution was dried over magnesium sulfate. The solvent was evaporated and the resulting product was purified on silica gel using ethyl acetate/chloroform system (**U1**–**U8**, see Supplementary data).

3.4. Enzymatic studies

The rates of inhibition of human neutrophil elastase (Biocentrum, Poland) and chymotrypsin (Calbiochem) was measured in a 0.1 M HEPES, 0.5 M NaCl, 0.03% Triton X-100 (pH 7.5); inhibition of trypsin (Sigma–Aldrich) was measured in 0.1 M HEPES, 0.01 M $CaCl_2$, pH 7.5. All enzymes were assayed using fluorogenic substrates: MeO-Suc-Ala-Ala-Pro-Val-AMC (Ex. 350 nm, Em. 460 nm, Calbiochem) for human neutrophil elastase, Suc-Ala-Ala-Pro-Phe-AMC (Ex. 350 nm, Em. 460 nm, Calbiochem) for chymotrypsin, and Bzl-Arg-AFC (Ex. 380 nm, Em. 505 nm, Sigma–Aldrich) for trypsin. The inhibitory activity was measured by incubation method under pseudo-first order conditions as described.[9] The standard deviation for presented values is the mean of three independent experiments and does not exceed 10%. All measurements were performed using Spectra Max Gemini XPS microplate reader (Molecular Devices).

3.4.1. Library screening

The screening of inhibitory activity of prepared libraries was done by the incubation method. A stock DMSO solution of each inhibitor mixture was diluted with an assay buffer to obtain concentration in a range from 2.5 μM to 62.5 nM of tested compounds. Next, 50 μl of the inhibitor solution was incubated with 100 μl of enzyme solution for 30 min at 37 °C. Final human neutrophil elastase concentration was 0.01 U. Then, 50 μl of the substrate buffered solution (240 μM, MeO-Suc-Ala-Ala-Pro-Val-AFC) was added into each well. The final concentration of DMSO in all samples was kept at 2.5%. The control sample contained only buffers with 2.5% DMSO. The measurement of inhibition level was read over 15 min in triplicate. The percent of inhibition was calculated in reference to the control sample.

3.4.2. Single inhibitor activity assay

The stock DMSO solution (10 mM) of the inhibitor was diluted with the assay buffer in the range between 5 μM and 5 nM. Fifty microliters of the inhibitor was incubated with 100 μl of the enzyme solution for 30 min at 37 °C. Final enzyme concentrations were as follows: 0.01 U (human neutrophil elastase), 1.25 nM (chymotrypsin), and 2.5 nM for trypsin. After 50 μl of the substrate buffered solution (240 μM, MeO-Suc-Ala-Ala-Pro-Val-AFC; 8 μM, Suc-Ala-Ala-Pro-Phe-AMC, and 400 μM—Bzl-Arg-AFC) was added into each well. To the enzyme–inhibitor solution 50 μl of substrate was added and the progress of the enzymatic reaction was monitored for 15 min. Measurement of each inhibitor concentration was repeated independently three times. The IC_{50} and $k_{obs}/[I]$ values were calculated as described previously.[9]

3.5. Cell culture

Human gingival fibroblasts (HGF-s) were derived from the tissue cultures of patients with healthy periodontium tissues undergoing extraction procedure. The gingival biopsies were provided by the Department of Dental Surgery of Wroclaw Medical University. The experiments were conducted in accordance with the requirements of the Bioethics Commission of Wroclaw Medical University. The human gingival fibroblasts were isolated from healthy gingival tissues according to the procedure described previously.[24]

HGF's were grown in Dulbecco's modified Eagles' medium (Sigma) supplemented with 10% fetal bovine serum and glutamine with penicillin/streptomycin (Sigma) in 25-cm^2 flasks (Nunc). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For experimental purposes, the cells were removed by trypsinization (0.25% Trypsin–EDTA, Sigma).

Human lung adenocarcinoma cell line (A549) was obtained from ATCC. The cells were grown in DMEM (Lonza) with addition of 10% fetal bovine serum (BioWhittaker, Lonza) and supplemented with antibiotics (penicillin/streptomycin, Lonza). For the experiments the cells were removed by trypsinization (0.25% Trypsin–EDTA solution, Sigma) and washed with PBS (IITD, PAN, Poland). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

3.6. Cell viability assay

Stock solutions of elastase inhibitors were prepared in DMSO and stored at −86 °C. On the day of the treatment, solutions of tested compounds were prepared in cell culture medium at different concentrations. Control cell cultures were treated with the equivalent amount of DMSO only. The MTT assay (Sigma) was used to test mitochondrial metabolic function. Cells were seeded into 96-well microculture plates at 1×10^4 cells/well and allowed to attach overnight. After incubation with various concentrations of the inhibitors, the assay was performed according to the manufacturer's protocol. The absorbance was measured using a multiwell scanning spectrophotometer at 570 nm (TECAN, Infinite 200). The results were expressed as the percentage of viable cells relative to untreated control cells. The cytotoxicity test was performed after 48 h of incubation with inhibitors. The average values were plotted as concentration of chemical versus percentage growth relative to control (± one standard deviation, indicated by error bars).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.008.

References

1. Wątorek, W.; Farley, D.; Salvesen, G.; Travis, J. *Adv. Exp. Med. Biol.* **1988**, *240*, 23.
2. Stockley, R. A. *Am. J. Respir. Crit. Care Med.* **1999**, *160*, 49.
3. Heutinck, K. M.; ten Berge, I. J.; Hack, C. E.; Hamann, J.; Rowshani, A. T. *Mol. Immunol.* **2010**, *47*, 1943.
4. Ioachimescu, O. C.; Stoller, J. K. *COPD* **2005**, *2*, 263.
5. Sato, T.; Takahashi, S.; Mizumoto, T.; Harao, M.; Akizuki, M.; Takasugi, M.; Fukutomi, T.; Yamashita, J. *Surg. Oncol.* **2006**, *15*, 217.
6. Machovich, R.; Himer, A.; Owen, W. G. *Blood Coagul. Fibrinolysis* **1990**, *3*, 273.
7. Oleksyszyn, J.; Powers, J. C. *Biochemistry* **1991**, *30*, 485.
8. (a) Sieńczyk, M.; Oleksyszyn, J. *Curr. Med. Chem.* **2009**, *16*, 1673; (b) Sabidó, E.; Tarragó, T.; Giralt, E. *Bioorg. Med. Chem.* **2010**, *18*, 8350; (c) Sabidó, E.; Tarragó, T.; Niessen, S.; Cravatt, B. F.; Giralt, E. *ChemBioChem* **2009**, *10*, 2361.
9. (a) Oleksyszyn, J.; Powers, J. C. *Methods Enzymol.* **1994**, *244*, 423; (b) Senten, K.; Daniëls, L.; Van der Veken, P.; De Meester, I.; Lambeir, A. M.; Scharpé, S.; Haemers, A.; Augustyns, K. *J. Comb. Chem.* **2003**, *5*, 336.
10. Sieńczyk, M.; Oleksyszyn, J. *Tetrahedron Lett.* **2004**, *45*, 7251.

11. Jackson, D. S.; Fraser, S. A.; Ni, L. M.; Kam, C. M.; Winkler, U.; Johnson, D. A.; Froelich, C. J.; Hudig, D.; Powers, J. C. *J. Med. Chem.* **1998**, *41*, 2289.
12. Sieńczyk, M.; Oleksyszyn, J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2886.
13. Joossens, J.; Van der Veken, P.; Lambeir, A. M.; Augustyns, K.; Haemers, A. *J. Med. Chem.* **2004**, *47*, 2411.
14. Joossens, J.; Van der Veken, P.; Surpateanu, G.; Lambeir, A. M.; El-Sayed, I.; Ali, O. M.; Augustyns, K.; Haemers, A. *J. Med. Chem.* **2006**, *49*, 5785.
15. Boduszek, B.; Oleksyszyn, J.; Kam, C. M.; Selzler, J.; Smith, R. E.; Powers, J. C. *J. Med. Chem.* **1994**, *37*, 3969.
16. (a) Abuelyaman, A. S.; Jackson, D. S.; Hudig, D.; Woodard, S. L.; Powers, J. C. *Arch. Biochem. Biophys.* **1997**, *344*, 271; (b) Liz, M. A.; Fleming, C. E.; Nunes, A. F.; Almeida, M. R.; Mar, F. M.; Choe, Y.; Craik, C. S.; Powers, J. C.; Bogyo, M.; Sousa, M. M. *Biochem. J.* **2009**, *15*, 467.
17. Mahrus, S.; Craik, C. S. *Chem. Biol.* **2005**, *12*, 567.
18. Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synthesis* **1979**, 985.
19. Dömling, A.; Ugi, I. *Angew. Chem., Int. Ed.* **2000**, *39*, 3168.
20. Giovenzana, G. B.; Tron, G. C.; Di Paola, S.; Menegotto, I. G.; Pirali, T. *Angew. Chem., Int. Ed.* **2006**, *45*, 1099.
21. Passerini, M. *Gazz. Chim. Ital.* **1921**, *56*, 126.
22. Sieńczyk, M.; Lesner, A.; Wysocka, M.; Łęgowska, A.; Pietrusewicz, E.; Rolka, K.; Oleksyszyn, J. *Bioorg. Med. Chem.* **2008**, *16*, 8863.
23. Sieńczyk, M.; Kliszczak, M.; Oleksyszyn, J. *Tetrahedron Lett.* **2006**, *47*, 4209.
24. Saczko, J. Polish Patent P 3812045, 2008.