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New potent cathepsin G phosphonate inhibitors

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ABSTRACT

Cathepsin G is an enzyme with dual chymotrypsin and trypsin-like specificity. As a leukocyte proteinase it is involved in the early stages of the immune response. In this work the synthesis and inhibitory activity of diaryl phosphonic-type irreversible cathepsin G inhibitors are described. Modification of the lead structure *Z*-Phg^{P}(OPh)$_2$ (**1**) (k_{obs}/I = 91 M^{-1} s^{-1}) in phenyl ester moieties followed by incorporation of the basic functional group into the aromatic side chain yielded highly potent cathepsin G inhibitor *Z*-(4-guanidine)Phg^{P}(OC$_6$H$_4$-4-*S*-Me)$_2$ (**12**) with the apparent second-order inhibition value at 15,600 M^{-1} s^{-1}. Further elongation of the obtained compound by tripeptide resulted in the inhibitor Ac-Phe-Val-Thr-(4-guanidine)Phg^{P}(OC$_6$H$_4$-4-*S*-Me)$_2$ (**19**) with the highest k_{obs}/I value ever reported in literature (256,000 M^{-1} s^{-1}).

1. Introduction

Cathepsin G (CG) is a member of polymorphonuclear leukocyte (PMN)-derived serine proteases which along with human neutrophil elastase and proteinase-3 is released upon leukocyte activation. Its physiological role in the living organism is mainly associated with early immune response[1] but its biologic activity is not limited to this event. Examples of the broad actions of cathepsin G include angiotensin I to angiotensin II conversion[2] and degradation of several extracellular matrix proteins such as collagen, laminin and elastin.[3] However, prolonged and excessive enzymatic action of cathepsin G leads to serious tissue damage and the propagation of inflammation.[4] Uncontrolled activity of cathepsin G activates gelatinase A (MMP-2) which may facilitate tumor invasion and angiogenesis.[5] Increased proteolysis must be precisely controlled by a system of endogenous inhibitors such as serpins and macroglobulins.[6] In case of cathepsin G leukocytes can suppress or extremely diminish its inhibition. For example, serpins or macroglobulins can be inactivated by oxidation[7] or proteolysis.[8] Moreover cathepsin G, like other leukocyte-derived enzymes, is bound to the leukocyte membrane or extracellular matrix components after secretion.[9] In their bound form enzymes are less susceptible to inhibitor action especially by large endogenous proteinase inhibitors.[10]

α-Aminoalkylphosphonate diphenyl esters are low molecular weight irreversible inhibitors of serine proteases. The ester structure formed after reaction of α-aminophosphonate with the active site Ser195 OH mimics the transition state of peptide bond cleavage performed by proteases. Due to their complete lack of activity toward cysteine or threonine proteases and relatively small size α-aminophosphonates are attractive alternatives to large inhibitors.[11]

In 1991 data regarding the synthesis of several compounds active against specific members of the serine protease family including cathepsin G were published.[12] However the obtained phosphonic analogue of phenylalanine (*Z*-Phe^{P}(OPh)$_2$) (**1**) showed poor inhibitory activity against cathepsin G (k_{obs}/I = 76 M^{-1} s^{-1}). A structurally similar inhibitor—phosphonic analogue of phenylglycine (*Z*-Phg^{P}(OPh)$_2$) displays similar potency toward cathepsin G (k_{obs}/I = 91 M^{-1} s^{-1}) and was used in our approach.

Using *Z*-Phg^{P}(OPh)$_2$ as the starting structure three series of analogues were synthesized with the intention to select cathepsin G inhibitors. In the first group modifications were introduced into the side chain α-aminophosphonate. Our previous results showed that substitution of guanidine group into the *para* position of the phenylalanine side chain in the structure of chromogenic peptidic substrate (in the position P$_1$) increased specificity (k_{cat}/K_M) to cathepsin G by 10-fold was achieved.[13] Since α-aminophosphonate aromatic esters interact with serine proteases in a substrate like manner we decided to introduce several basic substituents into the *para* position of *Z*-Phg^{P}(OPh)$_2$ side chain or even replace the whole side chain yielding phosphonic analogues of arginine or lysine.

From the first group the most active compound contained a 4-guanidine group was selected and was further modified within the phenyl ester rings. The following groups were introduced in the *para* position of esters: methyl, ethyl, methoxy, methylthionyl,

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isopropyl, naphtyl, *tert*-butyl, chloro and 1,1,3,3-tetramethylbutyl. The most potent cathepsin G inhibitor of this series was ester with methylthionyl moiety. The obtained structure was further modified by N-terminal elongation with Thr, Val-Thr and Ac-Phe-Val-Thr. The crystal structure of human cathepsin G in complex with Suc-Val-Pro-Phe^{P}(OPh)$_2$ has been reported.[14] At the bottom of the S1 specificity pocket Glu226 residue (chymotrypsinogen numbering) is located and, positively charged equatorial edge of phenyl ring from benzyl substituent of Phe^{P} is interacting with negatively charged carboxylate group of Glu226. The Glu226 carboxylate allows the accommodation of Lys side chain in the S1 pocket. Our mapping studies with the new chromogenic substrates are in agreement with these crystallographic data.

Recent reports by de Garavilla et al. regarding the synthesis and crystal structure of β-ketophosphonate dual inhibitors of cathepsin G and human chymase reveal that cathepsin G has a two hydrophobic binding sites (S1 and S3/S4) able to accommodate large hydrophobic naphthalene groups.[15] Again this was in good correlation with our previous data regarding chromogenic substrates where the Phe residue in position P4 displayed the highest specificity parameter.

2. Results and discussion

Inhibitory activity of the synthesized molecules against cathepsin G, along with HPLC and MS analyses results are presented in Tables 1–3. For the series of Z-protected phosphonic amino acid analogues (compounds **1**–**8**) the highest inhibitory potency was observed for the compound bearing the guanidine group at the *para* position of the side chain phenyl ring (**4**). The influence of this group on the inhibitory activity overcomes all other residues introduced at this position. Analogue **2** with an amino group introduced into the side chain at the *para* position displayed significantly higher activity when compared to the starting structure **1**. This suggests that the presence of –NH– group in this position is crucial for forming the network of bonds between the inhibitor molecule and the enzyme. The poor inhibitory properties of *Z*-(4-amidino)Phg^{P}(OPh)$_2$ seems to confirm this hypothesis. Low potency of phosphonic analogues of lysine, arginine and homoarginine (**5**–**7**) is most probably due to the relatively high flexibility of their aliphatic side chains (compounds **6** and **8** within 30 min of incubation with cathepsin G were able to inactivate only 5% of the enzyme whereas no inhibition was observed in the case of analogue **7**). These data correlate well with our previous results regarding the design of new chromogenic substrates of cathepsin G using combinatorial chemistry methods where tetrapeptide Ac-Phe-Val-Thr-Phe(*p*-guanidine) with amide of 5-amino-2-nitrobenzoic acid (ANB-NH$_2$) attached at C-terminus (served as a chromophore) displayed the highest specificity constant (k_{cat}/K_M).[13]

Significant improvement of cathepsin G inhibitory activity of **4** was achieved after introduction of substituents at the *para* position of phenyl ester rings. With only two exceptions (**15**, **16**) the inhibitory potency was enhanced and the highest increase was observed when methylthionyl was introduced (compound **12**). Such a substitution yielded inhibition greater than 30 times that of compound **4** with a k_{obs}/I value at 15,600 M^{-1} s^{-1}. Also, the introduction of methoxy groups (compound **11**) into this position increased inhibitor potency (over 10 times as compared to the parent compound) displaying k_{obs}/I = 7100 M^{-1} s^{-1}. Substitution of the phenyl ring of the ester moiety by bulky groups such as naphthyl (**16**) and 1,1,3,3-tetramethylbutane (**15**) decreased the inhibition strength by twofold (**16**) or made the compound almost inactive (**15**).

Elongation of structure 12 with a peptide chain highly increased the inhibitory activity against cathepsin G (Table 3). Any N-terminal extension by Thr (**17**), Val-Thr (**18**), and Ac-Phe-Val-Thr (**19**) considerably increased inhibitory activity. These kinetic inhibition data are well explained by reported X-ray structure and underline significant role of Glu226 carboxylate at the bottom of S1 specificity pocket of cathepsin G. Again, these results correspond very well with our previously published data regarding cathepsin G chromogenic substrate development.[13,16] The obtained tetrapeptide Ac-Phe-Val-Thr-Phg^{P}-(OC$_6$H$_4$-4-*S*-Me)$_2$ (**19**), appeared to be an extremely potent peptidic cathepsin G inhibitor with k_{obs}/I = 256,000 M^{-1} s^{-1} and to our knowledge, this compound displays the highest potency toward cathepsin G ever reported in the literature.

Selectivity of action of compounds **1**, 4, **11**, **12**, **17–19** was determined using cathepsin G related serine proteases such as bovine α-chymotrypsin, bovine β-trypsin and human thrombin (Table 4).[17–20] Structures **1**, **4**, **11** and **12** were more potent inhibitors of related proteases than cathepsin G. Introduction of peptidic chain into the inhibitor structure increased their selectivity of action. The highest influence on related enzymes activity of obtained inhibitors was observed for trypsin. Compound **17** was only two times more active against cathepsin G than against trypsin. Introduction of longer peptide chain (two or three amino acids, compounds **18** and **19**, respectively) lead to increased selectivity (12-fold increase for structure **18**, and 20-fold for **19**) if compared to trypsin. Compounds **17**, **18** and **19** were more potent toward cathepsin G than toward α-chymotrypsin and human thrombin, where 30 times (compound **17**) and 250 times (compound **19**) higher activity was observed.

Further investigation in this field may lead to more potent and selective inhibitors. The next challenge in this area is the complete structure activity relationship (SAR) as well as the synthesis of pure diastereomers of the most potent inhibitors. This would open a way for further in vivo studies using new potent cathepsin G inhibitors.

3. Materials and methods

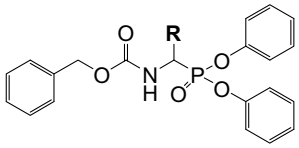
3.1. Chemical synthesis

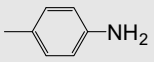
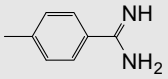
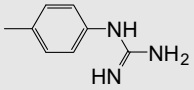
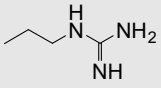
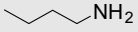
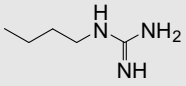
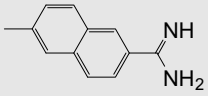
For the synthesis of α-aminophosphonates the amidoalkylation reaction was applied.[21] Condensation of triphenyl phosphite, benzyl carbamate and an aldehyde in glacial acetic acid resulted in α-aminoalkyphosphonate aromatic esters as the racemic mixtures (Table 1). Lead structure (**1**) was obtained using benzaldehyde. Compound **4** was obtained using a method described previously.[22] For the synthesis of compound **2** 4-nitrobenzaldehyde was used and the obtained *Z*-(4-NO$_2$)Phg^{P}(OPh)$_2$ was further reduced using $SnCl_2$ in ethyl acetate.[23] For the synthesis of **5**, **6** and **7** Phth-protected aldehydes were applied.[24,25] Guanylation of *Z*-(4-NH$_2$)Phg^{P}(OPh)$_2$ as well as compounds **5** and **6** was performed using *S*-ethyl-*N*,*N*'-di(Boc)-isothiourea in the presence of $HgCl_2$.[26] For the synthesis of **8** 6-carbonitrile naphthalene-2-al was prepared starting from dimethyl naphthalene 2,6-dicarboxylate and used in modified amidoalkylation reaction to obtain the desired α-aminophosphonate diphenyl ester **8**.[27,28] For the synthesis of α-aminoalkylphosphonates with substituted phenyl ester rings (**9**–**16**, Table 2) our previously described method was applied.[26]

3.2. Peptide synthesis

A benzyloxycarbonyl group was removed from *Z*-(4-guanidine(Boc)$_2$)Phg^{P}(OC$_6$H$_4$-4-*S*-Me)$_2$ (234 mg, 0.28 mmol) (**12**) by hydrogenolysis in methanol in the presence of 0.1% Pd/C resulting

Table 1
Inhibition of cathepsin G by *Z*-protected α-aminoalkylphosphonates diphenyl esters



Compound	R	MW calculated/obtained	t_R[a] (min)	*I* (μM)	k_{obs}/I[b] (M^{-1} s^{-1})
1		473.5/474.7	25.16	97	**91**
2		488.5/489.6	24.74	52	300
3		515.5/515.5	23.32	112	30% Inhibition[c]
4		530.5/531.5	22.72	47	412
5		482.5/483.5	15.12	99	74
6		454.5/455.3	15.56	221	5% Inhibition[c]
7		496.5/497.1	14.92	250	No Inhibition
8		569.6/570.2	28.11	112	5% Inhibiton[c]

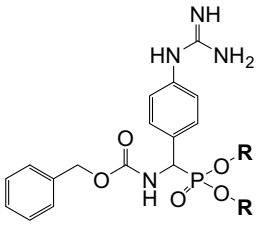
[a] HPLC retention time with a linear gradient 20–95% B (A: 0.1% TFA; B: 80% acetonitrile in A) within 45 min applied.
[b] The apparent second-order inhibition.
[c] Percent of inhibition after 30 min of incubation.

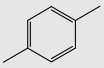
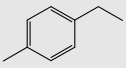
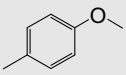
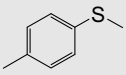
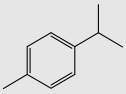
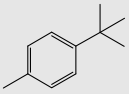
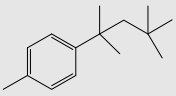
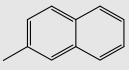
H_2N-(4-guanidine(Boc)$_2$) $Phg^P(OC_6H_4$-4-*S*-Me$)_2$ with 97% yield. Further peptide synthesis was performed in solution. H_2N-(4-guanidine(Boc)$_2$)$Phg^P(OC_6H_4$-4-*S*-Me$)_2$ (187 mg, 0.27 mmol) and Fmoc-Thr(*t*-Bu)-OH (118 mg, 0.27 mmol) were dissolved in 3 ml of dimethylformamide (DMF) and (1-*N*-hydroxybenzotriazol (HOBt) (40 mg, 0.27 mmol) was added. The solution was cooled in an ice bath and diisopropylocarbodiimide (DIPCI) (45 μl, 0.27 mmol) was added. The reaction mixture was stirred at −5 °C for 3 h and then overnight at room temperature. After the reaction was completed 15 ml of ethyl acetate was added and washed with 10% sodium bicarbonate, 5% citric acid and brine. After drying the volatile components were evaporated under reduced pressure affording protected dipeptide Fmoc-Thr(*t*-Bu)-(4-guanidine(Boc)$_2$)$Phg^P(OC_6H_4$-4-*S*-Me$)_2$ (**17A**) in 78% yield. The Fmoc group from **17A** was removed using 20% piperidine in DMF and product **17B** (yield 98%) was obtained. Final complete deprotection was achieved using a trifluoroacetic acid (TFA)/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v/v/v) leading to product **17**.[29] Further elongation was performed by applying the same procedure. The dipeptide **17B** was elongated with Val (Fmoc-Val-OH was applied) and Fmoc-Val-Thr(*t*-Bu)-(4-guanidine(Boc)$_2$)-$Phg^P(OC_6H_4$-4-*S*-Me$)_2$ (**18A**) was obtained (72%). The removal of Fmoc and *t*-Bu groups resulted peptide **18** with 93% yield. Finally Ac-Phe-OH was coupled to peptide **18B** giving fully protected peptide Ac-Phe-Val-Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-(OC_6H_4-4-*S*-Me$)_2$ (**19A) (**67% yield). The final step of the synthesis was a removal of side chain protecting groups from Thr and (4-guanidine(Boc)$_2$)Phg^P by TFA/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v/v/v) leading to Ac-Phe-Val-Thr-(4-guanidine(Boc)$_2$)$Phg^P(OC_6H_4$-4-*S*-Me$)_2$ (**19**). The purity of final products 17–**19** was verified by HPLC and the structure was confirmed by MS analysis. The HPLC and MS analyses results are summarized in Table 3.

3.3. HPLC analysis

Purity of individual compounds was examined using the RP-HPLC Pro Star system (Varian, Australia) equipped with a Kromasil

Table 2
Inhibition of cathepsin G by various esters of *N*-benzyloxycarbonylamino-(4-guanidino)Phg^{P}(O**R**)$_2$



Compound	R	MW calculated/obtained	t_R[a] (min)	I (μM)	k_{obs}/I[b] (M^{-1} s^{-1})
4		530.5/531.7	22.72	47	412
9		545.5/546.5	23.61	28	2820
10		558.6/560.7	24.98	48	1620
11		590.6/591.2	24.12	8	7100
12		622.7/623.4	25.34	4	**15,600**
13		614.7/615.6	26.28	24	2200
14		643.7/644.3	30.13	28	1150
15		754.9/755.1	32.67	115	3% Inhibition[c]
16		782.8/783.3	35.73	112	226

[a] HPLC retention time with a linear gradient 20–95% B (A: 0.1% TFA; B: 80% acetonitrile in A) within 45 min applied.
[b] The apparent second-order inhibition.
[c] Percent of inhibition after 30 min of incubation.

100 C_8 column (8 × 250 mm) (Knauer, Germany). A linear gradient from 20% to 95% B within 45 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm.

3.4. MS analysis

Mass spectra were recorded using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany). The alpha cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix.

3.5. Enzymatic studies

Cathepsin G isolated from human neutrophils was purchased from Biocentrum Kraków. All UV measurements were performed using Cary 3E spectrophotometer (Varian, Australia). The concentration of bovine β-trypsin stock solution was determined by titration with *p-nitrophenyl-p′*-guanidinebenzoate (NPGB). This trypsin stock solution was used for titration of SFTI-1, the mutual inhibitor of both bovine β-trypsin and cathepsin G. Solution of SFTI-1 was used to determine the concentration of the active form of cathep-

Table 3
Inhibition of cathepsin G by peptidyl derivates of *Z*-PhgP(4-guanidine)-$(OC_6H_4$-4-*S*-$Me)_2$

Compound	Sequence	MW calculated/found	t_R[a] (min)	k_{obs}/I[b] (M^{-1} s^{-1})
12	*Z*-Phg^P(4-guanidine)-$(OC_6H_4$-4-*S*-$Me)_2$	622.7/623.4	21.84	15,600
17A	Fmoc-Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-$(OC_6H_4$-4-*S*-$Me)_2$	1069.5/1069.7	28.73	No inhibition
17B	Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-$(OC_6H_4$-4-*S*-$Me)_2$	847.5/847.8	24.81	No inhibition
17	Thr-Phg^P(4-guanidine)-$(OC_6H_4$-4-*S*-$Me)_2$	603.2/604.3	19.23	52,300
18A	Fmoc-Val-Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-$(OC_6H_4$-4-*S*-$Me)_2$	1169.9/1170.0	30.21	No inhibition
18B	Val-Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-$(OC_6H_4$-4-*S*-$Me)_2$	947.8/948.1	26.02	No inhibition
18	Val-Thr-Phg^P(4-guanidine)-$(OC_6H_4$-4-*S*-$Me)_2$	702.8/703.1	20.18	152,000
19A	Ac-Phe-Val-Thr(*t*-Bu)-Phg^P(4-guanidine(Boc)$_2$)-$(OC_6H_4$-4-*S*-$Me)_2$	1137.0/1137.1	29.09	No inhibition
19	Ac-Phe-Val-Thr-Phg^P(4-guanidine)-$(OC_6H_4$-4-*S*-$Me)_2$	878.0/879.3	24.64	**256,000**

[a] HPLC retention time with a linear gradient 20–95% B (A: 0.1% TFA; B: 80% acetonitrile in A) within 45 min applied.
[b] The apparent second-order inhibition.

Table 4
Inhibitory activities against cathepsin G and related serine proteases

Compound	k_{obs}/I[a] (M^{-1} s^{-1})			
	Cathepsin G	α-Chymotrypsin	β-Trypsin	Thrombin
1	91	1050	No inhibition	No inhibition
4	412	30%[b]	11,300	1520
11	7100	430	13,200	2100
12	15,600	870	17,800	3070
17	52,300	1130	22,100	1560
18	152,000	1070	12,560	1170
19	256,000	1010	13,230	780

[a] The apparent second-order inhibition.
[b] Percent of inhibition after 30 min of incubation.

sin G. The inhibition constants were measured using two methods: the progressive curve method proposed by Tian and Tsou[30] and incubation method described by Oleksyszyn and Powers.[11]

The kinetics of the reaction between irreversible inhibitors (**12**–**14**) and cathepsin G were analyzed by continuous measurements of the loss of enzyme activity in the presence of chromogenic substrate under pseudo-first order conditions (inhibitor:enzyme molar ratio below 10).[30] Increasing concentrations of inhibitor (0.05–10 μM) in 50 μl aliquots was added and 10 μl of chromogenic substrate (Ac-Phe-Val-Thr-Gnf-ANB-NH_2, recently developed by our group[13]) at a concentration of 3.3 μM was mixed in a cuvette containing 0.1 M Tris–HCl buffer, pH 7.5, supplied with 500 mM NaCl to final volume of 1.54 ml. The reaction was initiated by addition of 25 μl of cathepsin G (2 μM) and the release of chromophore was monitored at 405 nm for 20 min. Each measurement was repeated and five to eight different concentrations of inhibitor were tested.

When the progress of reaction was too slow to be measured by the method described above (compounds **1**–**8** and **11**) a different approach was used.[11] In this case 20 μl of cathepsin G was added to 480 μl of assay buffer containing 50 μl of tested inhibitor (at 5–200 μM) yielding a final inhibitor concentration from 50 nM to 50 μM (the enzyme:inhibitor molar ratio was maintained over 10). At specific intervals 25 μl of reaction mixture was sampled and the enzyme residual activity was determined. The aliquots were added to 1.48 ml of the buffer followed by addition of 10 μl of 3.3 μM chromogenic substrate (Ac-Phe-Val-Thr-Gnf-ANB-NH_2). Product release was measured over a period of 3 min.

The $t_{1/2}$ values for the inhibition reaction were obtained from plots of $\ln(v_t/v_0)$ versus time. The pseudo-first order inhibition constant k_{obs} was calculated from equation $t_{1/2} = 0.693/k_{obs}$.[11] The inhibitory activity of synthesized compounds is expressed as the apparent second-order inhibition rate constant (k_{obs}/I). For compounds (**3**, **6**, **8**, **13**, **14**) that poorly inhibited cathepsin G within 30 min, the percentage of inhibition was determined.

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