Development of Irreversible Diphenyl Phosphonate Inhibitors for Urokinase Plasminogen Activator

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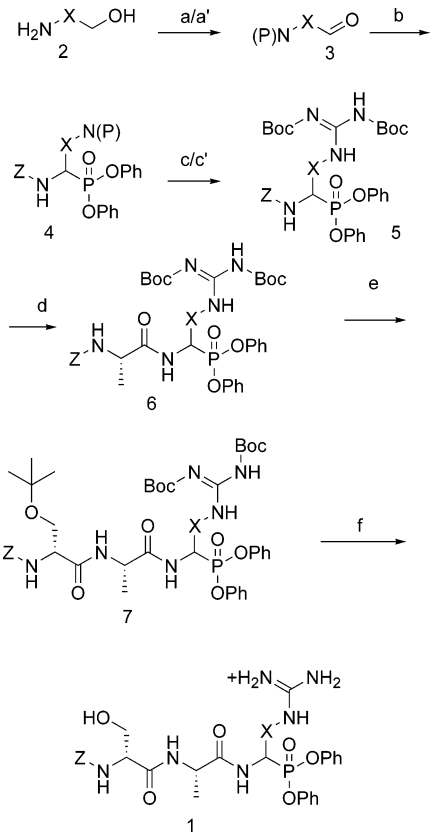
Abstract: In this letter we report the synthesis and biochemical evaluation of selective, irreversible diphenyl phosphonate inhibitors for urokinase plasminogen activator (uPA). A diphenyl phosphonate group was introduced on the substratelike peptide Z-D-Ser-Ala-Arg, and modification of the guanidine side chain was investigated. A guanylated benzyl group appeared the most promising side chain modification. A k_{app} value in the 10^3 M^{-1} s^{-1} range for uPA was obtained, together with a selectivity index higher than 240 toward other trypsin-like proteases such as tPA, thrombin, plasmin, and FXa.

Angiogenesis is the formation of new blood vessels from preexisting vasculature and is believed to be critical for tumor growth and metastasis. Tumors above a diameter of 2 mm^3 need a blood supply to grow. This blood supply is also important for the metastasis process. When a tumor switches to the angiogenetic phenotype, it starts producing growth factors.[1−3] These growth factors activate endothelial cells of the vasculature bed and stimulate them to grow toward the tumor. The next step requires breakdown of the extracellular matrix (ECM). It was proved that the degradation of ECM by proteases such as matrix metalloproteases (MMP's) is extremely important in angiogenesis.[4,5] MMP's are activated by plasmin, and this enzyme degrades several components of ECM, including fibrin, laminin, and fibronectin. Plasmin is activated from its zymogen, plasminogen, by urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), both of which are serine proteases. It is known that while tPA is mainly involved in the fibrinolysis process, uPA is more important in angiogenesis and is activated by binding to its receptor (uPAR).[6−9] The interaction with uPAR induces proliferation and migration responses, with both responses being unrelated to the serine protease activity.

Research suggests that uPA is important in tumor development. Experiments with knock-outs of all the components of the uPA system in mice show a reduction in tumor growth and metastasis. Moreover, high levels of uPA are related to poor prognosis in human cancers.[7] Experiments also prove that the deletion of the uPA system in mice has no consequences for viability.[6] Hence, the inhibition of uPA and/or the interaction with its receptor is considered to be an important approach for finding antiangiogenic agents.[8]

Scheme 1. Synthesis of Compounds **1**[a]



[a] (a) (i) Di-*tert*-butyl dicarbonate, (ii) Dess−Martin oxidation; (a′) (i) phthalic anhydride, Et_3N, toluene, 90 °C; (ii) Swern oxidation; (b) benzyl carbamate, triphenyl phosphite, $Cu(OTf)_2$, DCM; (c) (i) TFA, (ii) *N*,*N*′-bis(*tert*-butoxycarbonyl)-1-guanylpyrazole, MeCN; (c′) (i) $NH_2NH_2 \cdot H_2O$, THF, (ii) *N*,*N*′-bis(*tert*-butoxycarbonyl)-1-guanylpyrazole, MeCN; (d) (i) H_2/Pd, (ii) Z-Ala-OH, TBTU, DMF; (e) (i) H_2/Pd, (ii) Z-D-Ser(OtBu), TBTU, DMF; (f) TFA. (TBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate).

Two kinds of inhibitors for uPA are reported in the literature. One group consists of peptides and peptide derivatives, characterized by a basic function for a specific ionic interaction with the carboxylate group of Asp-189 of the S1 site in the deepest pocket of the substrate binding groove of the enzyme.[6] Other inhibitors, such as amiloride, are small molecules with an arylamidine, arylguanidine, or acylguanidine template, capable of performing the same ionic interaction as the peptides.[7] The development of very selective inhibitors for uPA is, however, hampered by its close resemblance to trypsin-like serine proteases, and several inhibitors suffer from a poor selectivity toward tPA, plasmin, or thrombin.

In this paper we describe the development of potent and selective diphenyl phosphonate peptidic inhibitors for uPA. The diphenyl phosphonate group is known to react with the active site serine affording a stable phosphonylated and irreversibly inhibited enzyme.[10,11,13] To the best of our knowledge, these diphenyl phosphonate peptides are the first selective and irreversible uPA inhibitors described in the literature. An irreversible chloromethyl ketone peptide was reported but lacks selectivity.[14]

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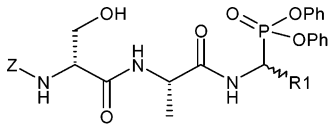
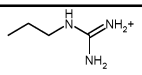
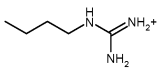
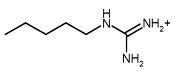
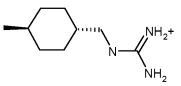
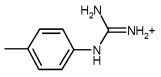
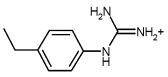
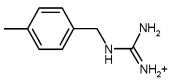
Table 1. Inhibition of Trypsin-Like Serine Proteases[a]



				IC_{50} (μM) or % inhibition at given concentration (μM)			
	R_1	IC_{50} (μM) uPA	k_{app} ($M^{-1}.s^{-1}$) uPA	tPA	Plasmin	Thrombin	FXa
1a		4.10 ± 0.01*	$1.4 \times 10^1 \pm 0.1 \times 10^1$	46% @ 250 (~60)**	15% @ 250 (>60)	10% @ 2.5 96% @ 250 (>0.6)	6% @ 250 (> 60)
1b		0.061 ± 0.001	$1.77 \times 10^3 \pm 0.08 \times 10^3$	50% @ 2.5 (~40)	10.8 ± 0.3 (300)	0.50 ± 0.02 (8)	65 ± 4 (1100)
1c		0.25 ± 0.01	$3.8 \times 10^2 \pm 0.1 \times 10^2$	19% @ 2.5 100 % @ 250 (>10)	39% @ 250 (>1000)	12% @ 2.5 100% @ 250 (>10)	13% @ 250 (>1000)
1d		1.1 ± 0.2	$1.40 \times 10^2 \pm 0.09 \times 10^2$	41% @ 250 (>200)	32.2 ± 0.8 (>28)	40% @ 250 (> 200)	77% @ 250 (<200)
1e		1.6 ± 0.02	$2.0 \times 10^2 \pm 0.2 \times 10^2$	53% @ 250 (~150)	46% @ 2.5 (~1.50)	44% @ 250 (>150)	56 % @ 250 (<150)
1f		0.057 ± 0.002	$6.7 \times 10^3 \pm 0.4 \times 10^3$	14± 1 (240)	22 ± 2 (380)	30 ± 4 (530)	79± 3 (1400)
1g		8.4 ± 0.4	$3.3 \times 10^1 \pm 0.2 \times 10^1$	43% @ 250 (>30)	45% @ 250 (~30)	20% @ 250 (>30)	44 % @ 250 (>30)
	Amiloride	107 ± 4		>>250 (>>2)	23% @ 250 (> 2)	>>250 (>>2)	17% @ 250 (>2)

[a] (*) standard error on the fit; (**) selectivity index: IC_{50}/IC_{50} uPA.

Tamura et al. reported the biological activity of Z-D-Ser-Ala-Arginal as an uPA inhibitor with an IC_{50} value of 19 nM and a selectivity ratio of more than 130 toward tPA.[15] The pharmacokinetic properties of this compound revealed a short plasma half-life following oral or subcutaneous administration.[7,15] Our experience with the diphenyl phosphonate group as an inhibitory moiety in peptide inhibitors for dipeptidylpeptidases prompted us to substitute the aldehyde group of Z-D-Ser-Ala-Arginal for an irreversibly inhibiting diphenyl phosphonate group.[10] The use of irreversible inhibitors could be very useful for further investigation of the usefulness of uPa inhibitors in antiangiogenetic settings. In addition, we optimized the arginine moiety by incorporating various linear or cyclic guanylated side chains at the P1 position.[12]

The synthetic pathway is shown in Scheme 1. *tert*-Butyl carbamate (Boc) amino alcohols **2d**−**g** were used as the starting materials for cyclic side chain compounds **1d**−**g**. When these amino alcohols were not commercially available, they were prepared from the corresponding amino acids by reduction with $LiAlH_4$ after Boc protection. A Swern[16] or Dess−Martin[17] oxidation to aldehydes **3d**−**g** followed by an amidoalkylation with benzyl carbamate and triphenyl phosphite afforded protected diphenyl phosphonate amino acids **4d**−**g** as racemic mixtures.[11,13,18−21] The amidoalkylation reaction, as originally described by Oleksyszin,[21] was slightly modified: higher yields were obtained when the usual solvent AcOH was substituted with dichloromethane, and copper triflate was employed as a catalyst. Treatment with TFA followed by conversion of the amine into a Boc-protected guanidine with *N*,*N*′-di(Boc)guanylpyrazole furnished the guanylated phosphonates **5d**−**g**.[22] The Z-protecting group was removed by hydrogenolysis, and the resultant free amine was coupled by the use of TBTU with Z-Ala-OH to obtain **6d**−**g**.[23] The Z-protecting group was again removed, and the protected peptides **7d**−**g** were obtained by coupling with Z-D-Ser-(tBu)-OH. The Boc protecting groups were removed with TFA. Final compounds **1d**−**g** were isolated as the corresponding TFA salt. The Boc protected amino alcohols could not be used as starting materials for compounds **1a**−**c**. Aldehydes **3a**−**c** cyclized easily, and the amidoalkylation gave low yields and/or afforded pyrrolidine phosphonate esters or piperidine phosphonate esters.[10,24] To avoid this reaction, the synthetic pathway was slightly modified, and a phthalimide protection was

used for compounds **2a**−**c**. After amidoalkylation in AcOH, the phthalimide moiety was removed with hydrazine. This deprotection unfortunately gave low yields due to the instability of the diphenyl phosphonate group. Aldehyde **3b** can also be prepared through acid hydrolysis of the *N*-phthalimido-protected 4-aminobutyraldehyde diethylacetal.

The diphenyl phosphonate tripeptides **1a**−**g** were evaluated for their ability to inhibit various trypsin-like serine proteases.[15] The IC_{50} values were calculated for uPA and four other trypsin-like serine proteases involved in the blood coagulation cascade (tPA, thrombin, plasmin, FXa). Potent and selective irreversible inhibitors of uPA were discovered. Compounds **1b** and **1f** were the most potent inhibitors for uPA with IC_{50} values around 60 nM. The results are summarized in Table 1. Amiloride was used as the standard uPA inhibitor.

Compound **1b** containing the substratelike arginine side chain showed poor selectivity toward thrombin. Compound **1f** with a guanylated benzyl side chain showed a selectivity ratio (IC_{50} enzyme/IC_{50} uPA) of more than 200 for all enzymes tested.

After diluting the inhibited enzyme with substrate solutions, no rise in activity of uPA was observed, and it was concluded that these inhibitors were irreversible inhibitors of uPA. The k_{app} values for uPA were calculated from the progress curves. Further optimization of Z-D-Ser-Ala-amino-(4-guanylphenyl)ethanephosphonate diphenyl ester (**1f**) as an uPA inhibitor is in progress.

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Supporting Information Available: Experimental, biochemical, and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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