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4-Aminoarylguanidine and 4-Aminobenzamidine Derivatives as Potent and Selective Urokinase-type Plasminogen Activator Inhibitors

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Abstract—The structure-based design of potent and selective urokinase-type plasminogen activator (uPA) inhibitors with 4-aminoarylamidine or 4-aminoarylguanidine S1 binding groups, is described.

The serine protease, urokinase-type plasminogen activator (uPA) plays a key role in several biological processes, including tissue remodeling, cell migration, and matrix degradation.[1–3] High levels of uPA are associated with many different tumor types and a strong correlation between the level of uPA and poor prognosis has been noted.[1] Furthermore, a small, moderately potent uPA inhibitor, B428 (**1**; $K_i = 0.21\ \mu M$), has also shown efficacy in models of prostate and breast cancer.[4–7] Based on these promising findings there has been an increased interest in the development of potent and selective uPA inhibitors as cancer therapeutics.

The inhibitor **2** (Fig. 1), identified from screening a library of amidine-bearing heterocycles, was previously developed into a series of potent and selective uPA inhibitors based on the 5-carboxamidine substituted benzimidazole or indole, (e.g., **3**) heterocycle.[8] It was discovered that the S1 binding group could be replaced by a simple amide-linked 4-aminobenzamidine group (e.g., **4a**) without a loss in affinity toward uPA. The more facile synthesis of **4a** prompted the development of this lead in parallel to the development of **2**. This report discusses the results of a structure-based design approach toward the optimization of **4a** and contrasts these inhibitors to those based on benzimidazole **2** or indole **3**.[8]

The key reaction in preparing the inhibitors described in Table 1 is the formation of an amide bond between an arylamine and a salicylic acid derivative (Scheme 1).

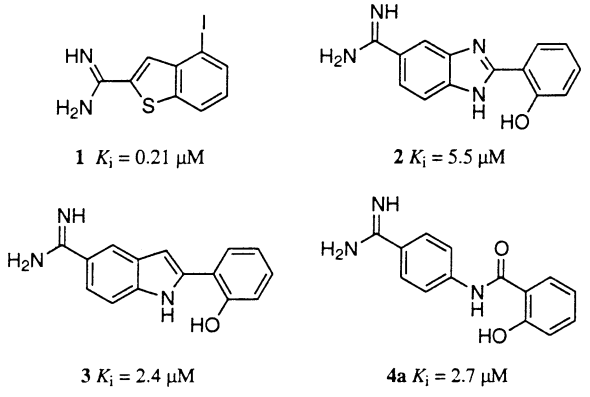
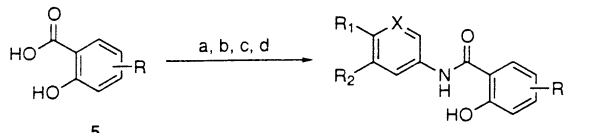


Figure 1. Selected inhibitors of uPA.



4a-n X = C, R_1 = C(=NH)NH_2, R_2 = H
6a-b X = C, R_1 = C(=NH)NH_2, R_2 = F
7a-b X = C, R_1 = C(=NH)NH_2, R_2 = Cl
8a-r X = C, R_1 = NHC(=NH)NH_2, R_2 = H
9a-e X = N, R_1 = NHC(=NH)NH_2, R_2 = H

Scheme 1. (a) Ac_2O, H_2SO_4; (b) $(COCl)_2$, cat. DMF, EtOAc; (c) arylamine, DMA; (d) NH_4OH (or satd $NaHCO_3$), THF.

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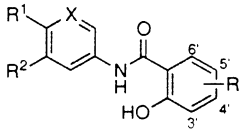
Table 1. Inhibition profiles of amidine- and guanidine-based uPA inhibitors



No.	R^1	X	R^2	R			K_i (μM)				
				3′	4′	5′	uPA	t-PA	Factor Xa	Thrombin	Plasmin
4a	Am	CH	H	H	H	H	2.7	10	12	22	90
4b	Am	CH	H	H	H	NO_2	44	94	99	185	N.D.
4c	Am	CH	H	H	H	Br	8.0	21	15	29	N.D.
4d	Am	CH	H	H	H	Me	2.6	6.9	9.4	14	N.D.
4e	Am	CH	H	H	H	OMe	3.1	9.4	5.2	12	N.D.
4f	Am	CH	H	H	Cl	H	6.0	24	2.8	9.5	27
4g	Am	CH	H	H	Me	H	1.0	3.6	2.3	10	6.0
4h	Am	CH	H	H	OEt	H	0.65	2.0	37	34	N.D.
4i	Am	CH	H	Cl	H	H	0.60	1.5	1.0	1.0	N.D.
4j	Am	CH	H	Me	H	H	1.7	3.9	5.7	8.0	18
4k	Am	CH	H	Ph	H	H	8.5	53	188	120	N.D.
4l	Am	CH	H	H	-Naphthyl-		0.19	1.8	12	23	4.0
4m	Am	CH	H	Br	H	Me	0.15	0.36	0.44	0.28	4.4
4n	Am	CH	H	I	H	Me	0.080	0.24	0.46	0.38	2.9
6a	Am	CH	F	I	H	Me	0.27	1.7	0.52	0.41	5.5
7a	Am	CH	Cl	I	H	Me	6.0	256	115	245	>75
8a	Gu	CH	H	H	H	H	24	>95	104	>450	>75
8b	Gu	CH	H	H	H	NO_2	>75	>95	>470	315	N.D.
8c	Gu	CH	H	H	H	Br	27	>95	>470	>450	N.D.
8d	Gu	CH	H	H	H	Me	13	>95	>470	>450	>75
8e	Gu	CH	H	H	H	OMe	20	>95	>470	>450	N.D.
8f	Gu	CH	H	H	Cl	H	27	>95	>470	>450	N.D.
8g	Gu	CH	H	H	Me	H	5.5	>95	198	>450	>75
8h	Gu	CH	H	H	OEt	H	10	>95	>470	>450	>75
8i	Gu	CH	H	Cl	H	H	42	>95	>470	>450	N.D.
8j	Gu	CH	H	Me	H	H	60	>95	350	>450	N.D.
8k	Gu	CH	H	Ph	H	H	>75	>95	>470	>450	N.D.
8l	Gu	CH	H	H	-Naphthyl-		0.30	>95	>470	>450	283
8m	Gu	CH	H	Br	H	Me	8.0	287	151	125	205
8n	Gu	CH	H	I	H	Me	8.5	>563	>470	>450	>75
8o	Gu	CH	H	Br	-Naphthyl-		1.6	>95	>470	>450	>75
8p	Gu	CH	H	I	-Naphthyl-		4.6	>95	>470	>450	>75
8q	Gu	CH	H	OMe	-Naphthyl-		0.25	181	13	205	55
8r	Gu	CH	H	O^{i}Pr	-Naphthyl-		0.23	>563	240	>450	75
9a	Gu	N	H	H	-Naphthyl-		0.085	338	55	43	>75
9b	Gu	N	H	Br	-Naphthyl-		0.22	506	235	330	>75
9c	Gu	N	H	I	-Naphthyl-		0.70	225	68	310	>75
9d	Gu	N	H	OMe	-Naphthyl-		0.28	150	>78	195	>75
9e	Gu	N	H	O^{i}Pr	-Naphthyl-		0.18	300	88	>450	>75

N.D., not determined; Am, $C(=NH)NH_2$; Gu, $NHC(=NH)NH_2$.

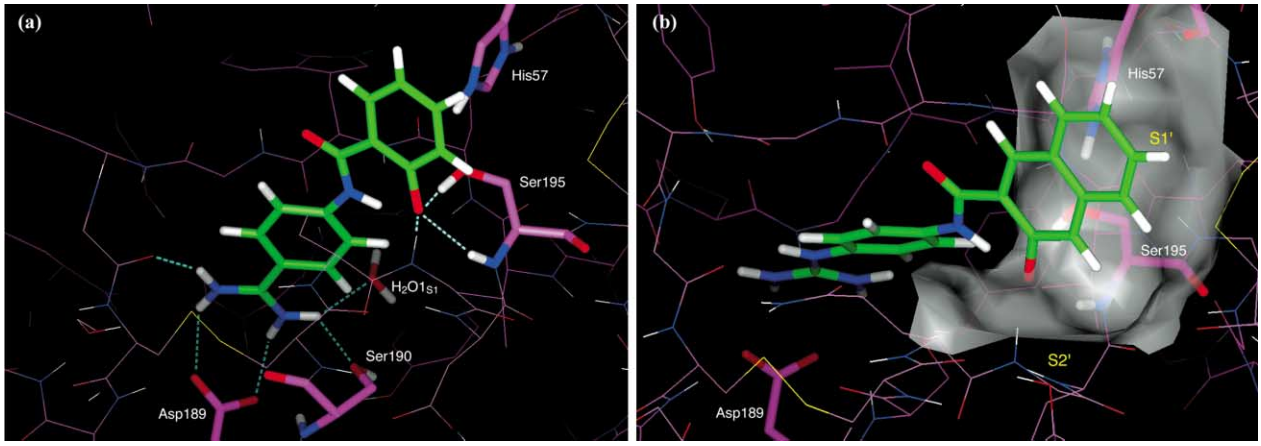
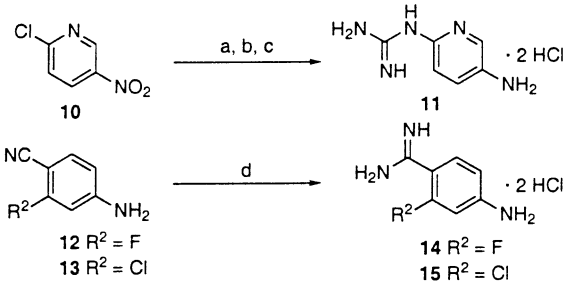


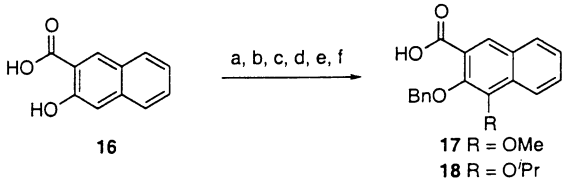
Figure 2. (a) X-ray structure of uPA–**4a** complex. The inhibitor co-binds with a water molecule H_2O1_{S1} near Ser190 and also makes direct hydrogen bonds with Asp189. The hydroxyl group binds in the oxyanion hole forming H-bonds to $N\alpha_{Ser195}$ and $N\alpha_{Gly193}$ and $O\gamma_{Ser195}$. (b) X-ray structure of uPA–**8l** complex. The position *ortho* to the hydroxyl group is directed toward S2′ affording an avenue for further optimization.

Clean and efficient amide bond formation required the mixing of the acetyl (or benzyl) protected salicylic chloride derivative and the aryl amine in dimethylacetamide (DMA).[9] The aryl amines that have not been previously reported were prepared according to Scheme 2. The acids were either commercially available, readily prepared from commercial materials through standard halogenation procedures, or synthesized according to Scheme 3.[10]

The lead molecule **4a** can display two modes of binding depending on the serine protease.[11] One binding mode is similar to uPA–**2** and involves the formation of an array of unusually short (<2.3 Å) hydrogen bonds between the inhibitor hydroxyl group, Ser195 and a water molecule trapped in the oxyanion hole. The alternative mode of binding, observed in the uPA–**4a** complex places the hydroxyl group in the oxyanion hole, hydrogen bonding to Ser195 (Fig. 2a). The inhibitors were tested for affinity toward a panel of five serine proteases and the results are shown in Table 1. Substitutions at 5′, analogues **4b**–**e**, did not enhance potency toward any of the enzyme targets despite the fact that this substitution affects the pK_a of the phenol group. This result was consistent with the results found in a similar series based on the benzimidazole scaffold **2**.[8] Potency increased with select groups at either the 3′ or 4′ sites (e.g., **4h**,**i**) or naphthyl analogue **4l**. The most noteable inhibitors were the 3′-halo derivatives (**4m**,**n**) that enhanced potency by 5- to 34-fold toward uPA and by up to 79-fold toward thrombin. This trend compares well with that observed for similar substitutions on the inhibitor **2**.[8] However, in contrast to **2**, the 3′-phenyl derivative **4k** was a weak uPA inhibitor. This difference is most likely due to the oxyanion-hole binding mode of these inhibitors in uPA, which directs the 3′-substituent into residue Ser195 and Cys42 rather than toward the S1′ subsite. The size of the group adjacent to the hydroxyl is therefore limited to sterically small groups in this mode of binding.



Scheme 2. (a) Guanidine, ^{t}BuOH, 120 °C; (b) H_2, 10% Pd/C, MeOH; (c) HCl, dioxane; (d) Me_3Al, NH_4Cl.



Scheme 3. (a) MeOH, 18 N H_2SO_4; (b) $MgCl_2$, $(CH_2O)_n$, Et_3N; (c) BnBr, Cs_2CO_3; (d) H_2O_2, H_2SO_4; (e) alkyl-I, Cs_2CO_3; (f) KOH.

Despite the enhanced activity toward uPA in some analogues, especially **4n**, the selectivity against the other enzymes is only marginal. In the series of inhibitors based on **2** a small halo group at the 6-position adjacent to the amidine, designed to displace the water molecule (H_2O1_{S1}) that co-binds in S1 (Fig. 2a), introduced excellent selectivity against Ala190 proteases (tPA, factor Xa, plasma-kallikrein, and thrombin).[12,13] Displacement of the water molecule in the Ala190 proteases leaves the amidine in an environment, lacking a full complement of hydrogen bonding partners thus leading to reduced potency. The Ser190 proteases can compensate for the loss of the water molecule by the formation of a full hydrogen bond between the amidine and $O\gamma_{Ser190}$. The introduction of a chlorine group at the 6-position on the most potent analogue **4n** was found to generate with **7a** a compound with good selectivity toward uPA and against the Ala190 enzymes. Unfortunately, the potency of **7a** toward uPA was reduced by up to 126-fold. The smaller fluorine group in analogue **6a** only marginally improved selectivity against one enzyme, tPA. In the analogue series based on **2**, the chlorine analogue was also found to be superior to fluorine in selectivity, but importantly, potency toward uPA was not significantly reduced by the chlorine substitution.[13] The alternative binding mode between the two series of molecules is once again believed to be the reason for the subtly different effects in potency and selectivity.

It has been reported that aryl guanidines favor binding to uPA but not plasmin, tissue-type plasminogen activator (tPA) and other proteases.[14,15] The basis for this preference is not fully understood but may involve the Ser190/Ala190 difference within S1. However, plasmin, a Ser190 enzyme, prefers lysine in S1 and also disfavors guanidines in general, so other factors must contribute to this selectivity preference. Replacement of the amidine on inhibitors generated a complementary series of inhibitors, **8a**–**m**. As predicted, potency against all the Ala190 enzymes and plasmin was markedly reduced but in the majority of examples, the uPA activity was also reduced especially for the promising 3′-halides (**8m**,**n**). One analogue, **8l**, however, was equipotent to its amidine counterpart **4l** and highly selective, >500-fold, against all the anti-targets.

A high-resolution X-ray structure of **8l** in uPA was solved (Fig. 2b) and reveals that the mode of binding is very similar to that of the uPA–**4a** complex. The site adjacent to the hydroxyl, like the 3′-position in analogues **4a**–**n**, is directed toward residue 195 and is presumably the reason for the reduced activity of the bromine and iodine analogues **8o** and **8p**, respectively.

It was reasoned that oxygen linked groups may afford better results by providing a sharp directional change. Analogues **8q**,**r** were indeed found to retain excellent potency and selectivity toward uPA.[16] This result now opens an avenue for S2′ directed modifications to further increase potency. Finally, substitution of the phenyl S1 binding ring for a pyridyl ring on the optimal inhibitors **8l** and **8o**–**r**, analogues **9a**–**e**, respectively, led to a slight increase in potency toward uPA. Inhibitor **9a**

($K_i = 85$ nM) is the most potent and selective inhibitor in the series and has several sites or vectors directed towards the S1–S2′ binding regions for further optimization.

In summary, a series of amidine-based, nonselective uPA inhibitors and a series of guanidine-based, selective inhibitors of uPA have been developed. The inhibitors display a unique mode of binding toward uPA, which results in a subtly different SAR to a closely related series of uPA inhibitors. The accumulated structural information and SAR for the selective inhibitors now provides a solid platform for further optimization in the S1–S2′ subsites.

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9. The salicylic acids were first acetylated by dissolution in acetic anhydride and a catalytic amount of 18 N H_2SO_4. The product after concentration and drying, was dissolved in ethyl acetate and treated with oxalyl chloride (10 equiv) and a few drops of DMF. After 60 min, the mixture was concentrated, dissolved in DMA and treated with the arylamine (1.2 equiv). After 17 h, the mixture was treated with concentrated ammonium hydroxide. The product was collected by filtration. Purification if necessary was carried out by HPLC methods. Intermediates having hydroxyl groups protected as benzyl ethers were coupled using the same procedure except without the acetylation step. Benzyl removal was performed by hydrogenation over 10% palladium on carbon catalyst (1 atm).
10. Preparation of **17** and **18**: A methanolic solution of **16** was treated with HCl (g) at 0 °C for 15 min, refluxed for 2 h, and then concentrated. The methyl ester product was formylated (see ref 17) to yield an equimolar mixture of the 4-formyl and 8-formyl isomers. The desired product methyl, 4-formyl-3-hydroxynaphthoic acid was soluble in hot methanol and could be separated by filtration from the methyl, 8-formyl-3-hydroxynaphthoic acid isomer. The product was treated with 1 equiv of Cs_2CO_3 in DMF for 60 min followed by 1 equiv of benzyl bromide to give the benzyl ether. The ether, dissolved in methanol: 18 N H_2SO_4 (25:1), was stirred with 35% (w/w) hydrogen peroxide solution (6.0 equiv) for 19 h and concentrated. Further treatment of the product with 1.1 equiv of Cs_2CO_3 in DMF for 60 min followed by addition of the necessary alkyl halide (3.0 equiv) gave the desired benzyl protected ether product. Treatment with KOH in MeOH removed the methyl ester to give the desired acid.
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