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2-(2-Hydroxy-3-alkoxyphenyl)-1*H*-benzimidazole-5-carboxamidine Derivatives as Potent and Selective Urokinase-type Plasminogen Activator Inhibitors

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Abstract—The development of potent and selective urokinase-type plasminogen activator (uPA) inhibitors based on the lead molecule 2-(2-hydroxy-3-ethoxyphenyl)-1*H*-benzimidazole-5-carboxamidine (**3a**) is described.

Urokinase-type plasminogen activator (uPA) is a trypsin-like serine protease that has been implicated in the processes of tumor cell invasion and metastasis.[1] Studies involving the use of anti-uPA antibodies,[2] antisense oligonucleotides,[3] uPA −/−knockout mice,[4] and small molecule inhibitors,[5] all support an important role of uPA in tumor progression. Furthermore, high levels of uPA correlate with poor prognosis in several types of cancer.[1a] Potent and selective inhibitors of uPA may therefore be therapeutically useful drugs for the treatment of metastatic cancer.[6] In several earlier reports we described the discovery of a novel series of trypsin-like serine protease inhibitors (e.g., **1**–**3a**) that bind to the active site of trypsin-like serine proteases through the formation of a unique cluster of short (<2.3 Å) and ordinary range hydrogen bonds (Table 1).[7] This class of inhibitors displays broad spectrum inhibitory activity toward the family of trypsin-like serine proteases and affords excellent leads for the development of potent and selective inhibitors of uPA.

In this report, we describe the potency and selectivity optimization of lead inhibitor **3a** toward uPA.

Inhibitors **1** and **2** bind to the active site of uPA and span from subsite S1 to S1′.[7] In a similar manner, **3a** also binds in these two subsites (Fig. 1a) placing the 5-carboxamidine substituted heterocycle in the S1 subsite and directing the 3′-ethoxy group toward S1′. The latter group provides an opportunity for exploring the S1′ subsite to optimize potency and selectivity. Several analogues based upon **3a** were synthesized according to Scheme 1, and their inhibition profiles toward uPA and several other proteases are depicted in Table 1.[7] The key precursors required to synthesize the benzimidazole analogues are an arylaldehyde (e.g., **6**), and an aryl diamine (e.g., **7**–**9**) (Scheme 1).[7a] A Mitsunobu reaction was used to introduce the different 3′-alkoxy groups on salicylaldehyde **5**. The arylethers, **6**, were then oxidatively condensed with one of the aryldiamines **7**–**9** to furnish the benzimidazole heterocycle.[7,8] The benzyl group was removed and where necessary, the nitrile group was converted to an amidine using reported procedures.[7a] To prepare indoles **12a**–**c** the benzyl protected salicylaldehyde **5** was first converted to the alkyne **10**,[9] and then subjected to a palladium mediated coupling[10] with mesylaniline **11**[11] to yield the *N*-mesyl indole. A Mitsunobu coupling introduced the 3′-alkoxy group, which was then followed by removal of the mesyl and benzyl groups and conversion of the nitrile to an amidine to give the indoles **12a**–**c**.[7a]

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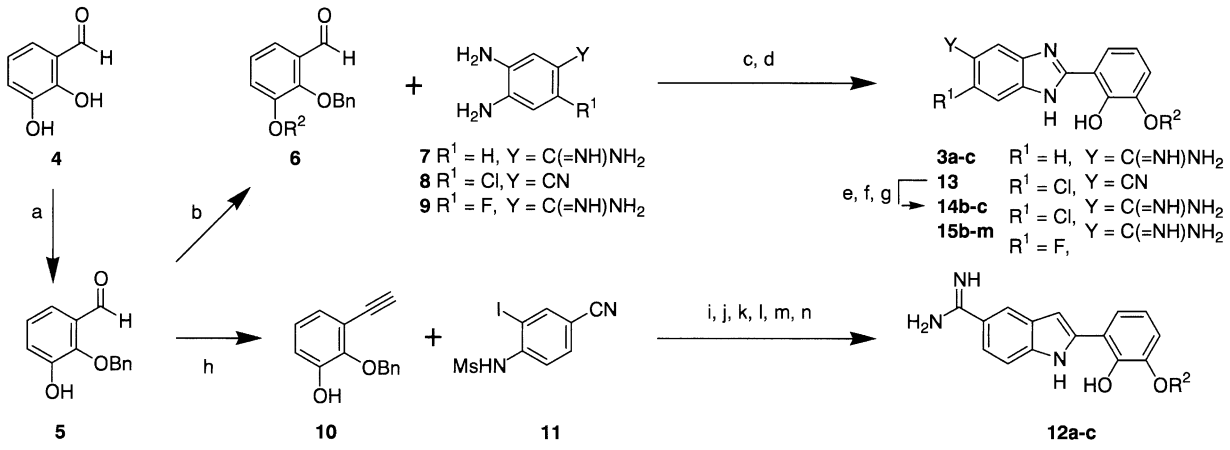
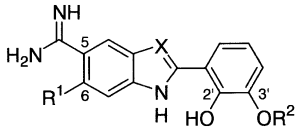
Inhibitors **1** and **2** bearing a 3′-phenyl ring reveal that a single atom change (benzimidazole to indole) can result in up to 50-fold greater affinity toward some enzymes.[7] In contrast, the indole analogues **12a–c**, which have 3′-alkoxy groups, only display up to 3-fold greater affinity than their benzimidazole counterparts, **3a–c**. The 3′-alkoxy groups are situated in close proximity to the cluster of hydrogen bonds and presumably introduce different effects to the phenyl groups found in **1** and **2**. These effects may be both electronic, exerted through the



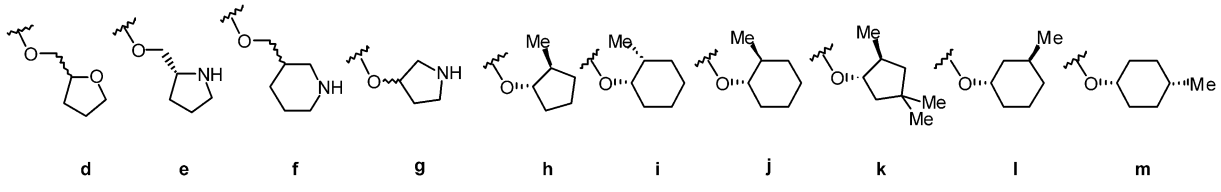
Scheme 1. (a) BnBr, Cs_2CO_3; (b) R^2OH, DEAD, PPh_3; (c) EtOH, $Na_2S_2O_3$, reflux; (d) H_2, 10% Pd/C, MeOH; (e) NH_2OH, EtOH, reflux; (f) Ac_2O, AcOH; (g) H_2, 10% Pd/C, MeOH; (h) K_2CO_3, Bestmann's reagent;[9] (i) $Pd(PPh_3)_2Cl_2$, Cu^II, DMF, TEA; (j) R^2OH, DEAD, PPh_3; (k) KOH, MeOH; (l) H_2, 10% Pd/C, MeOH; (m) NH_2OH, EtOH, reflux; (n) Zn, AcOH.

Table 1. Inhibition profiles of the inhibitors toward 5 trypsin-like serine proteases



Compd	R^1	X	OR^2	K_i (μM)				
				uPA	tPA	Factor Xa	Thrombin	Plasmin
1	H	N	Ph	0.40	1.0	2.1	15	5.0
2	H	CH	Ph	0.008	0.035	0.078	0.32	0.10
3a	H	N	OEt	0.70	3.2	6.8	31	1.6
3b	H	N	O^{i}Bu	0.25	1.0	2.4	15	1.3
3c	H	N	O(C_5H_9)	0.22	0.69	1.5	15	0.60
12a	H	CH	OEt	0.45	1.7	2.1	9.0	0.65
12b	H	CH	O^{i}Bu	0.18	0.88	1.7	7.5	0.65
12c	H	CH	O(C_5H_9)	0.19	0.59	0.99	7.5	0.31
14a	Cl	N	OEt	0.95	>75	>75	>75	1.6
14b	Cl	N	O^{i}Bu	0.55	16	34	90	2.2
14c	Cl	N	O(C_5H_9)	0.55	29	43	165	1.0
15a	F	N	OEt	0.29	>75	41	>75	1.8
15b	F	N	O^{i}Bu	0.19	61	31	23	2.7
15c	F	N	O(C_5H_9)	0.22	33	32	32	1.6
15d	F	N	[a]	0.08	45	13	20	4.2
15e	F	N	[a]	0.70	275	167	70	27
15f	F	N	[a]	0.46	263	125	50	21
15g	F	N	[a]	1.2	225	94	50	47
15h	F	N	[a]	0.032	44	34	20	0.85
15i	F	N	[a]	0.032	58	65	16	2.6
15j	F	N	[a]	0.011	29	34	11	1.1
15k	F	N	[a]	0.11	425	83	14	3.1
15l	F	N	[a]	0.18	150	38	22	3.0
15m	F	N	[a]	0.10	71	38	10	1.7

[a]OR^2 groups for **15d–m**



covalent bonds, or alternatively, spatial effects, such as hydrogen bonding interactions between the 3′-O atom and the hydroxyl group. For example, it has been proposed that the preferred binding conformer of the tautomeric benzimdazole, in the short hydrogen bond cluster, is that in which the nitrogen proximal to the hydroxyl group is protonated.[7b] Therefore, one reasonable possibility, is that the 3′-O in this series of inhibitors may well promote the more preferred tautomeric form of the benzimidazole, compared to the 3′-phenyl series, through the formation of hydrogen bonds between the protonated nitrogen, the hydroxyl group and the 3′-O. Since the indole heterocycle offered no significant advantage over the benzimidazole in this series of 3′-alkoxy analogues, the more easily synthesized benzimidazoles were the focus of future efforts toward inhibitors displaying greater potency and selectivity.

Selectivity toward uPA and against tissue-type plasminogen activator (tPA) can be significantly enhanced by the introduction of a chlorine or fluorine atom at the 6-position adjacent to the amidine.[10,12] The halogen substitution displaces a water molecule that is co-bound with the inhibitor in the vicinity of residue 190. In the enzymes that possess an Ala190 residue (tPA, factor Xa, and thrombin) the water molecule is an essential hydrogen bond partner for the amidine group and its displacement leads to a marked reduction in affinity toward these enzymes. The serine hydroxyl group in the Ser190 enzymes (e.g., uPA and plasmin) can compensate the loss of the water molecule, thereby maintaining affinity. Comparing the 6-chlorine and 6-fluorine substituted analogues (**14a–c** and **15a–c**, respectively) with their unsubstituted counterparts (**3a–c**) reveals that selectivity toward uPA and against the other enzymes was improved without a loss of affinity toward uPA. Despite the improved selectivity of **15a–c** compared to **3a–c**, the selectivity against plasmin was not improved since plasmin, like uPA, has a Ser190 residue.

The 3′-alkoxy substituent provides a convenient entry point into the S1′ subsite (Fig. 1a) to try and improve selectivity against plasmin. The X-ray structure of a catalytically inactive mutant of plasmin indicates that there are some differences within the S1′ region compared to uPA.[13] In plasmin, residue 41 is a phenylalanine whereas in uPA it is a smaller valine residue. The peptide backbone in the region of Phe41 is shifted by $\sim$2 Å inwards and the disulfide bridge between Cys42 and Cys58 is also moved inwards, leading to a smaller S1′ subsite in plasmin compared to uPA. Efforts to increase the steric bulk of the 3′-isobutyl group on the inhibitor, **15b**, in an attempt to exploit the larger S1′ subsite of uPA compared to plasmin were successful and yielded a series of inhibitors, **15d–m**, with improved plasmin selectivity. Analogues **15d–g**, indicated that secondary nitrogen atoms tended to give relatively weak uPA inhibitors. For example, the tetrahydrofurfuryl analogue **15d** was ten times more potent toward uPA than the corresponding pyrrolidine analogue **15e**. Bulky, hydrophobic, five- and six-membered rings (e.g., **15h–m**) yielded the most potent and selective analogues (e.g., **15h–j**). Multiple methyl groups on the cyclohexyl ring (e.g., **15k**) or moving the methyl group around the ring, analogues **15l** and **15m**, reduced affinity toward uPA and also, to a lesser extent, plasmin. The most selective inhibitor was *trans*-2-methylcyclohexanol **15j**, which had a $K_i = 11$ nM toward uPA and greater than 100-fold selectivity against plasmin and the other anti-targets. It was interesting to note that the *cis*-isomer (**15i**) displayed a similar inhibition profile to the *trans*-isomer (**15j**).

The complex of **15j** in uPA was solved (Fig. 1b and c) and compared to that of **15b**. The inhibitor **15j** binds more deeply in the S1 pocket than **15b**, leading to the displacement of two water molecules co-bound in S1. The short hydrogen bond cluster involving Ser195 is broken and new short hydrogen bonds form between the inhibitor oxygens and His57. The markedly different binding modes of **15b** compared to **15j** is surprising, and underscores how changes in inhibitor structure can have significant affects upon the mode of binding.

By taking advantage of small differences between uPA and other related proteases a series of potent and

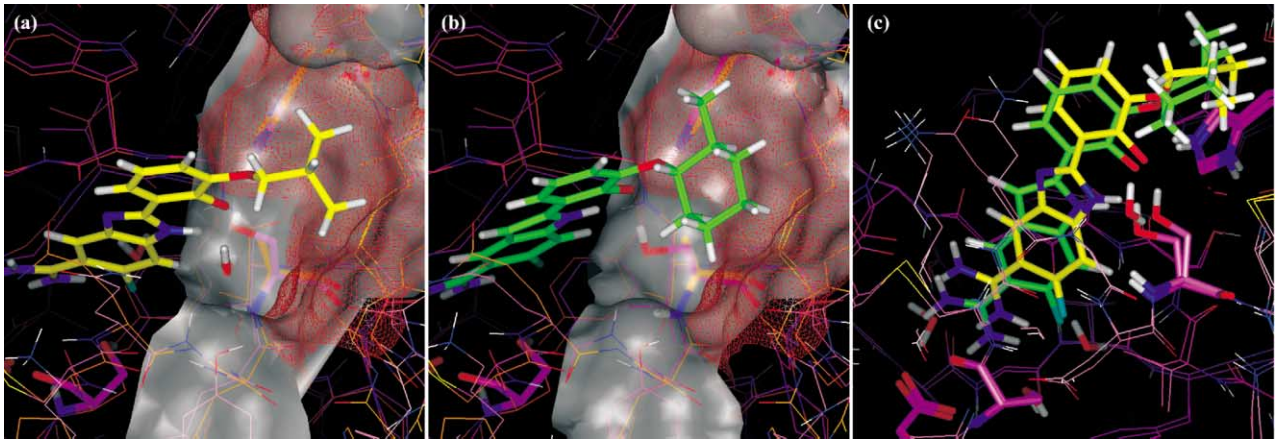


Figure 1. (a) uPA–**15b** complex. The plasmin surface is in red overlaid onto the uPA surface of S1′. The isopropyl group extends into S1′; (b) uPA–**15j** complex; (c) overlay of uPA–**15b** and uPA–**15j** complexes. In uPA–**15j**, the inhibitor has sunk deeper into S1 and displaced two water molecules that can be seen in the uPA–**15b** complex.

broadly selective uPA inhibitors have been generated. Two significant advances that improved potency and selectivity were the introduction of a fluorine atom adjacent to the amidine and also the substitution of the 3′-ethoxy group for a sterically larger alkyl ether. The inhibitors produced will likely provide unique opportunities for studying the role of selective uPA inhibitors in vitro and in vivo environments.

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8. 3,4-Diaminobenzamidine **7** was prepared as described in ref 7a. Preparation of 2-chloro-3,4-diaminobenzonitrile **8**: 2-chloro-4-fluorobenzonitrile was treated with potassium nitrate in 18 N sulfuric acid. The nitrated product was then treated with ammonium hydroxide to yield 2-chloro-4-amino-5-nitrobenzonitrile. Reduction over Pd/C catalyst under 1 atm hydrogen gave the desired diamine. Preparation of 2-fluoro-3,4-diaminobenzamidine **9**: 2,4-difluorobenzamidine was nitrated using potassium nitrate in 18 N sulfuric acid. The product was treated with ammonia to yield 2-fluoro-4-amino-5-nitrobenzonitrile and then reduced over Pd/C catalyst under 1 atm hydrogen to yield 2-fluoro-4,5-diaminobenzonitrile. Treatment with di-*tert*-butyldicarbonate (2 equiv) was followed by conversion of the nitrile to the amidine using standard procedures.[10] Removal of the BOC groups using HCl generated the 2-fluoro-3,4-diaminobenzamidine.
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11. 4-Aminobenzonitrile was treated with *N*-iodosuccinimide in acetic acid to yield 3-iodo-4-aminobenzonitrile. The aryl halide was then treated with mesyl chloride (2 equiv). The product was hydrolysed with potassium hydroxide to generate the *N*-mesyl-3-iodobenzonitrile **11**.
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