Fragment-Based Discovery of Mexiletine Derivatives as Orally Bioavailable Inhibitors of Urokinase-Type Plasminogen Activator[†]

Martyn Frederickson,* Owen Callaghan, Gianni Chessari, Miles Congreve, Suzanna R. Cowan, Julia E. Matthews, Rachel McMenamin, Donna-Michelle Smith, Mladen Vinković, and Nicola G. Wallis

Astex Therapeutics Ltd, 436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA, United Kingdom

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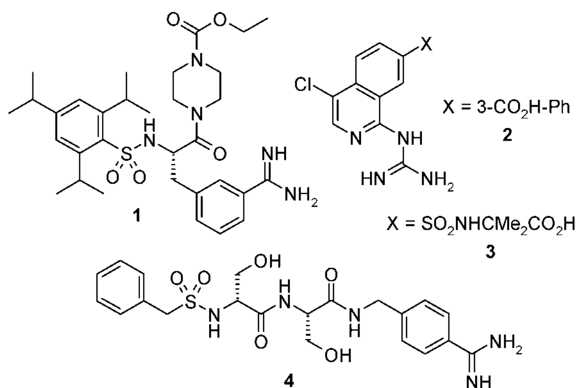
Abstract: Fragment-based lead discovery has been applied to urokinase-type plasminogen activator (uPA). The (*R*)-enantiomer of the orally active drug mexiletine **5** (a fragment hit from X-ray crystallographic screening) was the chemical starting point. Structure-aided design led to elaborated inhibitors that retained the key interactions of (*R*)-**5** while gaining extra potency by simultaneously occupying neighboring regions of the active site. Subsequent optimization led to **15**, a potent, selective, and orally bioavailable inhibitor of uPA.

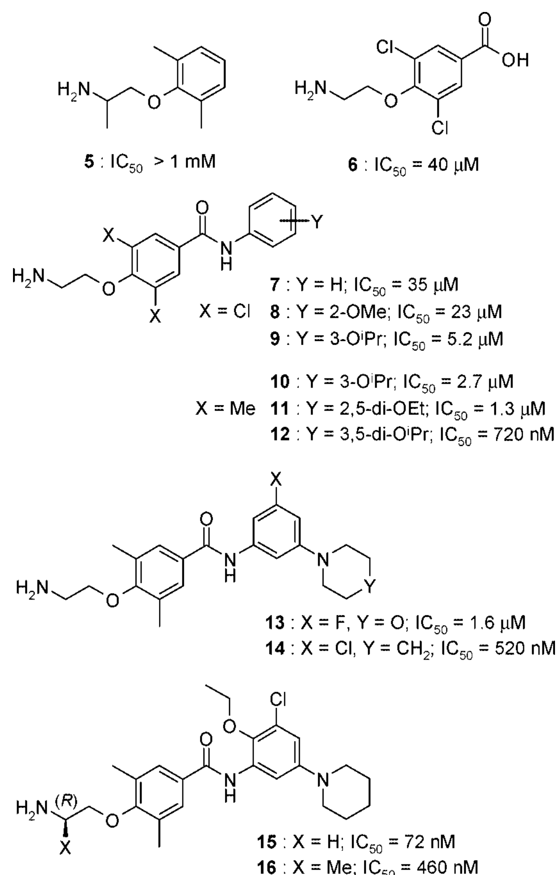
Urokinase-type plasminogen activator[1] (uPA[a]) is a trypsin-like serine protease[2] that, when bound to the extracellular membrane-associated urokinase-type plasminogen activator receptor[3] (uPAR), catalyzes the conversion of plasminogen to plasmin through the cleavage of the amide bond of an arginine/valine motif. Plasmin is responsible for a number of proteolytic processes that degrade various components of the extracellular matrix,[4] thereby triggering the induction of cell migration. As a result, uPA has been implicated in the progression of a variety of disease states associated with abnormal tissue destruction and cell infiltration including aortic aneurism,[5] multiple sclerosis,[6] and metastasis processes[7,8] in cancer.

Chart 1. Advanced Inhibitors of uPA



The active site of uPA[9] contains an aspartate residue (Asp189) within the deep S_1 pocket, which accommodates the side chain of the key arginine of plasminogen. Unlike other trypsin-like serine proteases that possess an adjacent hydrophobic alanine residue (e.g., thrombin and Factor Xa), the main binding cleft of uPA contains a more hydrophilic serine (Ser190) that effectively precludes the binding of hydrophobic functionalities that are commonly found in inhibitors of thrombin[10] and Factor Xa. Advanced inhibitors[11] of uPA (in the clinic or in preclinical studies) are thus dominated by classical arginine mimetics such as guanidines and amidines (Chart 1); such inhibitors are usually charged at physiological pH and accordingly have low oral bioavailability. We were thus interested in the discovery of novel inhibitors of uPA with less basic S_1 moieties that might overcome this problem.

Chart 2. Compounds **5**–**16**[a]



[a] Assay data quoted for compounds with $IC_{50} < 5$ μM are averages of two or more duplicate measurements.

Herein we disclose a novel series of weakly basic orally bioavailable inhibitors of uPA derived from mexiletine **5** (Chart 2). We describe briefly the X-ray crystallographic cocomplex of (*R*)-**5** with uPA, a key fragment hit from an X-ray crystallographic screen[12] against the enzyme, and outline the structure-aided development of the initial hit into more advanced, selective, and orally bioavailable inhibitors, as exemplified by compound **15**.

A fragment hit from X-ray crystallographic screening against uPA was the orally active drug mexiletine **5**. Despite having only relatively poor in vitro activity against uPA (IC_{50} > 1 mM), **5** had a very clear crystallographic binding mode (Figure 1a); although, being a racemate, only the (*R*)-

* To whom correspondence should be addressed. Phone: +44 (0)1223 226239. Fax: +44 (0)1223 226201. E-mail: m.frederickson@astex-therapeutics.com.

[†] Coordinates and structure factors for cocomplexes of urokinase-type plasminogen activator with compounds (*R*)-**5**, **6**, **9**, **11**, **14**, and **15** have been deposited in the Protein Data Bank under accession codes 2VIN, 2VIO, 2VIP, 2VIQ, 2VIV, AND 2VIW, respectively.

[a] Abbreviations: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; LE, ligand efficiency.

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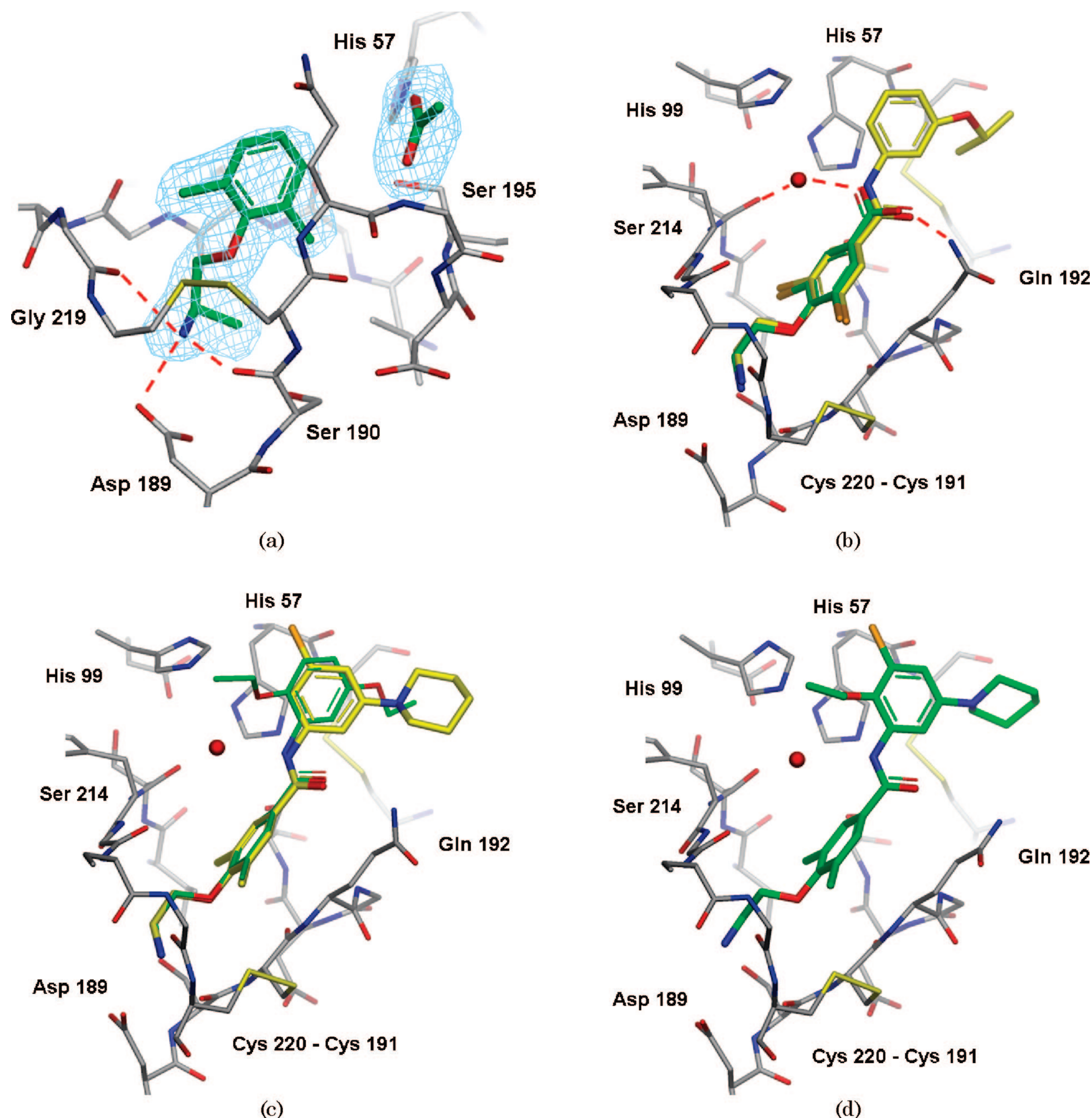

Figure 1. X-ray crystallographic cocomplexes of uPA with selected inhibitors: (a) with (*R*)-**5** (ligand electron density shown in light blue contoured at 3σ); (b) with **6** (green) showing **9** (yellow) overlaid for comparison; (c) with **11** (green) showing **14** (yellow) overlaid for comparison; and (d) with **15**. Hydrogen bonds are shown as red dashed lines and water molecules are shown as red spheres. The acetate anion in (b), (c), and (d) has been removed for clarity.

enantiomer of **5** was found to bind to the enzyme. The primary amine moiety of (*R*)-**5** binds to the carboxylate functionality of the aspartate group (Asp189) and to the backbone carbonyl of the serine residue (Ser190) that lie at the base of the S_1 pocket of the protein, with the pendant ethanolamine derived spacer and aromatic ring making substantial hydrophobic contacts with the residues that line the pocket. The amino functionality is also involved in a hydrogen bond with the oxygen of a neighboring glycine residue (Gly219). We simultaneously observed the presence of an acetate anion (from the crystallization buffer) in the X-ray structure located in the oxyanion hole of the protein (formed by the side chain of Ser195 and the three contiguous backbone NHs of Gly193, Asp194, and Ser195) close to the catalytic triad (Ser195, His57, and Asp102). Previous crystallographic studies of uPA have similarly highlighted the propensity of the sulfate anion[13] to bind in this location.

Despite its low ligand efficiency[14] (LE ~ 0.30 kcal mol^{-1} heavy atom^{-1}) when compared to simple benzamidines[15] (LE $\geq$ 0.50), we chose to develop (*R*)-**5** further as it is a known, highly water soluble, and orally bioavailable drug [$F_{\text{oral (man)}}$ = 90%]. Compound (*R*)-**5** was also attractive to us due to its relatively low pK_a of 9.2 when compared to many uPA inhibitors described previously, the basicity of the amine being lowered significantly by the presence of the β-oxygen atom in the side chain. The desire to reduce the pK_a of the Asp189 engaging functionality in uPA inhibitors to increase the likelihood of good oral bioavailability has been voiced previously.[13,15]

The clear binding mode of (*R*)-**5**, in conjunction with previously published structural data for a series of naphtha-

midines[16] that simultaneously occupy neighboring binding sites, strongly suggested that subsequent substitution at the 4-position of the aromatic ring would lead to derivatives with much improved potency. In initial experiments, solely for reasons of immediate commercial accessibility, we initially replaced the two *ortho*-methyl groups of **5** with chlorine atoms. We also chose to remove the angular methyl group in the ethanolamine side chain; as in the protein–ligand structure, this group was proximal to the Ser190 side chain (resulting in steric crowding in the S_1 pocket) and so was likely to be detrimental to affinity. We therefore prepared acid **6** together with a limited number (guided by known structural data and by virtual screening) of simple aromatic amides of which **7**, **8**, and **9** are representative members.

As we progressed, we observed an increase in in vitro potency (IC_{50}: **5** > 1 mM; **6**, 40 μM; **7**, 35 μM; **8**, 23 μM; **9**, 5.2 μM) and were able to rationalize this with reference to X-ray crystallographic structures of cocomplexes of the compounds with uPA (Figure 1b); in all cases, the amide (or acid) carbonyl was shown to form a key hydrogen bond with the protein (to the amidic side chain NH_2 of Gln192). The amides also formed a specific water-mediated hydrogen bond (between the amidic NH and the backbone carbonyl of Ser214). Additionally, the binding modes of the anilides (**7**, **8**, and **9**) showed the newly appended aromatic ring to make substantial contacts with a number of neighboring aromatic residues of the protein while one of the pair of chlorine atoms was involved in a soft interaction with a neighboring disulphide bridge (Cys191–Cys220) that forms part of the $S_{1\beta}$ subpocket.[9]

Having rapidly demonstrated clear SAR around the aniline ring of **7** and using precise structural data from a number of uPA crystallographic cocomplexes, we returned our attention to compounds that, like mexiletine, contained a pair of methyl groups in place of chlorine atoms. We were keen to fully exploit the SAR that we had observed, and aided by virtual screening, we prepared a limited number of *ortho*- or *meta*-substituted anilides, including **10**, **11**, and **12**. Once again, potency against uPA was shown to increase (IC_{50}: **10**, 2.7 μM; **11**, 1.3 μM; **12**, 720 nM), with the more highly substituted and lipophilic derivatives being the most effective inhibitors.

An additional beneficial effect of the incorporation of an amide moiety *para*- to the phenolic oxygen atom became apparent when the basicities of several of the derivatives were determined. Thus, **10** had a measured pK_a of 8.75, around half of a log unit lower than that of **5**. This effect is presumed to be due to the amide that, as a mesomeric electron withdrawing group, subtly enhances the basicity modifying effect of the side chain β-oxygen atom.

Also of particular interest was the effect of the 2-ethoxy group of **11**. The structure of the X-ray crystallographic cocomplex formed between uPA and **11** showed the combined substituents of the 2,5-disubstituted aniline to be readily accommodated by the protein. Given the improved potency of **12**, we also prepared a series of 3,5-disubstituted derivatives, with the intention to subsequently append a 2-EtO substituent onto the most promising scaffolds that we discovered.

Due to the limited commercial availability of 3,5-disubstituted anilines, the derivatives that we invested time in preparing were specifically chosen based upon the structural data that was available to us and on the results of docking experiments; amides **13** and **14** are key indicative examples. Morpholine derivatives such as **13** (IC_{50} 1.6 μM) were somewhat less potent than the analogous piperidines such as **14** (IC_{50} 520 nM). As the crystallographic structures of both **11** and **14** cocomplexed with uPA (Figure 1c) were highly complementary, we prepared the conjoined derivative **15** (so incorporating the 2-EtO group) as well as (*R*)-**16**, the corresponding specifically substituted angular methyl derivative of **15** [analogous to (*R*)-**5**].

The 2-ethoxy derivative **15** (measured pK_a of 8.75) is a potent inhibitor of uPA (IC_{50} 72 nM; LE = 0.31) and is comparable to **1**−**4** (Chart 1; reported K_i values of 410 nM,[17] 37 nM,[18] 12 nM,[19] and 20 nM,[7] respectively). As expected, (*R*)-**16** (IC_{50} 460 nM; LE = 0.27) had markedly less in vitro activity than **15**. The maintenance of LE as the series progressed is in accord with the ideas recently reported[20] by Hajduk. The X-ray crystallographic structure of the cocomplex of **15** with uPA (Figure 1d) highlighted a binding mode analogous to those of both **11** and **14**.

The selectivity of **15** for uPA was determined by reference to IC_{50} values (data not shown) measured against a panel of closely related proteases (plasmin, Factor VIIa, Factor Xa, tissue-type plasminogen activator, and trypsin). Amine **15** was found to be selective for uPA (>50-fold) over all but one of the enzymes but was only moderately so (3- to 4-fold) against trypsin, the enzyme to which (in terms of the similarity of the S_1 subsite) it is most closely related and over which selectivity for uPA is most difficult to obtain.[21] That such limited selectivity over trypsin was not an inherent property of all compounds akin to **15** was shown by reference to compound **14**, which showed somewhat greater selectivity for uPA (around 10-fold).

On the basis of the observed high potency and selectivity, the pharmacokinetic profile of compound **15** was studied in the rat. When dosed orally at 4.2 mg/kg, **15** showed well defined and reproducible PK data (Table 1); importantly, amine **15** had high oral bioavailablity [$F_{\text{oral (rat)}}$ = 60%]. The moderate clearance level, high volume of distribution, and long half-life (7½ hours) observed for **15** are consistent with those observed for mexiletine **5** in man.[22] Compound **15** thus displays many of the highly desirable molecular properties expected of a good "lead-like" molecule, including high potency and selectivity over a series of closely related proteases and, importantly, high oral bioavailability, a molecular property that has so far remained relatively elusive[23] in advanced inhibitors of this heavily studied therapeutic target.

Table 1. Pharmacokinetic Profile for **15** (in Rat)

Cl (mL/min/kg)[a]	V_d[a] (L/kg)	$t_{½}$[a] (hr)	F (%)[b]
43	27	7.5	60

[a] A 0.95 mg/kg i.v. dose (solution in 100% H_2O). [b] A 4.2 mg/kg oral dose (solution in 100% H_2O).

Selective optimization of side activities of biologically active compounds, the so-called SOSA approach,[24] has gained favor in recent years as a viable alternative to more traditional screening methods. The idea that screening small collections of structurally diverse drug molecules against new therapeutic targets can aid the discovery of new drugs has appealed to many, chiefly because the safety, pharmacology, and bioavailability profile of any hit so obtained has implicitly already been well studied in man. Mexiletine **5** is a Class Ib antiarrythmic licensed for use in patients with life threatening irregular heartbeat and may also be of use in the control of refractory pain. The findings outlined herein clearly demonstrate that the functionalities present in (*R*)-**5** are also pertinent in the development of orally bioavailable inhibitors of the clinically relevant serine protease uPA.

Fragment-based screening experiments against uPA have been reported previously, using both X-ray crystallography[13] and NMR techniques.[15] Our studies have now shown that X-ray

crystallographic screening[12] is an appropriate technique for the discovery of novel, low basicity fragment hits against uPA that can be successfully developed into more advanced lead-like compounds (such as **15**) with highly desirable physicochemical properties, including high oral bioavailability.

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Supporting Information Available: Experimental procedures for the synthesis of compounds **6**−**16**, in vitro assay protocols for determination of IC_{50} values against uPA, and X-ray crystallographic details for uPA cocomplexed with (*R*)-**5**, **6**, **9**, **11**, **14**, and **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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