Urokinase plasminogen activator as an anti-metastasis target: Inhibitor design principles, recent amiloride derivatives and issues with human/mouse species selectivity

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Abstract

The urokinase plasminogen activator (uPA) is a widely studied anticancer drug target with multiple classes of inhibitors reported to date. Many of these inhibitors contain amidine or guanidine groups, while others lacking these groups show improved oral bioavailability. Most of the X-ray co-crystal structures of small molecule uPA inhibitors show a key salt bridge with the side chain carboxylate of Asp189 in the S1 pocket of uPA. This review summarises the different classes of uPA inhibitors, their binding interactions and experimentally measured inhibitory potencies and highlights species selectivity issues with attention to recently described 6-substituted amiloride and 5-*N,N*-(hexamethylene)amiloride (HMA) derivatives.

The urokinase plasminogen activation system (uPAS)

The urokinase plasminogen activation system (uPAS) comprises the trypsin-like serine protease (TLSP) urokinase plasminogen activator (uPA), its cognate cell surface receptor (uPAR) and three endogenous serpin inhibitors, plasminogen activator inhibitors PAI-1, PAI-2 and PAI-3 (Fig. 1) (Andreasen et al. 2000; Croucher et al. 2008; Duffy & Duggan 2004; Salajegheh 2016; Ulisse et al. 2009). Once activated, uPA converts inactive plasminogen to plasmin, which then triggers downstream activation of multiple proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsins (Vassalli et al. 1991). Plasmin, whose activity is regulated by α_2 -antiplasmin (Hall et al. 1991) can also activate latent growth factors in the extracellular matrix (ECM) (Pedrozo et al. 1999). Under normal physiological conditions, tight control of the activation of these enzymes is required to facilitate the degradation of basement membrane (BM) and remodeling of extracellular matrix (ECM) components in an ordered manner. Increased uPA expression and activity promote tumour cell invasion into surrounding tissues by stimulating the breakdown of BM and ECM (Andreasen et al. 1997; Didiasova et al. 2014; Magill et al. 1999). Dysregulated activity of the uPAS is also implicated in tumour cell proliferation, migration and metastasis (Kugaevskaya et al. 2018; Mahmood et al. 2018; Zhang et al. 2018) and contributes to poor prognosis (Su et al. 2016) and progression in various cancers including melanoma (Cho et al. 2019), hepatocellular carcinoma (HCC) (Wei et al. 2019), lung adenocarcinoma (Zhu et al. 2020), neuroendocrine (Özdirik et al. 2020), oral (Wyganowska-Świątkowska & Jankun 2015), gastric (Brungs et al. 2017; Kaneko et al. 2003), ovarian (van der Burg et al. 1996), breast (Tang & Han 2013; Xing & Rabbani 1996), prostate (Kimura et al. 2020), colorectal (Yang et al. 2000) and bladder cancers (Hasui & Osada 1997; Iwata et al. 2019). In breast cancer, the uPAS has been identified as the most reliable independent prognostic marker of poor prognosis (Banyś-Paluchowski et al. 2019; Bouchet et al. 1994; Duffy et

al. 1988; Foekens et al. 2000; Harbeck et al. 2002). Small molecule inhibitors of the uPA-uPAR interaction (Bum-Erdene et al. 2020), the uPA protease domain (Buckley et al. 2018; Lee et al. 2004; Rockway et al. 2002) and PAI-1, whose overexpression is also correlated with pro-tumourigenic activity have been developed (Kubala & DeClerck 2019). In this review, we focus on small molecule inhibitors of the uPA serine protease domain as several studies have revealed that inhibition of uPA-mediated proteolysis can reduce tumour growth and metastasis in rodent models, supporting uPA protease activity as a potential anticancer drug target (Andreasen et al. 2000; Henneke et al. 2010; Matthews et al. 2011a; Ngo et al. 2011; Santibanez 2017; Su et al. 2016; Ulisse et al. 2009). For recent reviews on uPA-uPAR antagonists and PAI-inhibitors see (Yuan et al. 2021) and (Kubala & DeClerck 2019), respectively.

Fig. 1 The urokinase plasminogen activation system (uPAS) in tumour invasion and metastasis. BM: basement membrane, ECM: extracellular matrix, MMPs: matrix metalloproteinases, PAI: plasminogen activator inhibitors, uPAR: urokinase plasminogen activation receptor. Created with BioRender.com.

Structure of uPA

Human pro-uPA is a 53 kDa single-chain glycoprotein (Wun et al. 1982) consisting of 411 amino acids with glycosylation at Asn302 (Bansal & Roychoudhury 2006; Lenich et al. 1992), secreted by various normal and tumour cells (Blasi 1988). It comprises an N-terminal growth factor-like domain (residues 1-43) that binds to the cell-surface-anchored uPAR, a central kringle domain (residues 47-135), a common motif observed in related serine proteases (*e.g.* tissue plasminogen

activator (tPA) and thrombin) and a C-terminal serine protease catalytic domain (residues 159-411) bearing the catalytic triad His57, Asp102 and Ser195 (numbering after cleavage, Fig. 2) (Spraggon et al. 1995; Stepanova & Tkachuk 2002). A salt bridge between the side chain amino group of Lys16 and the side chain carboxylate of Asp194 stabilizes the oxyanion hole. Regions comprising the S1 specificity pocket and oxyanion hole are displaced in single-chain pro-uPA, leading to disruption of this salt bridge and explaining its low proteolytic activity (Hedstrom 2002). Cleavage of the Lys158-Ile159 bond by plasmin generates the active, disulfide-linked two-chain high molecular weight (HMW; residues 1-411) uPA (Kasai et al. 1985; Magill et al. 1999; Spraggon et al. 1995). Further cleavage of the Lys135-Ile136 bond in chain A yields soluble, low molecular weight (LMW; residues 136-411) uPA and an amino-terminal fragment (ATF; residues 1-135) (Stepanova & Tkachuk 2002). HMW and LMW uPA display similar activities towards plasminogen (Sato et al. 2002). Once activated, uPA converts inactive plasminogen into catalytically-active plasmin by hydrolysing its Arg561-Val562 peptide bond (Schuster et al. 2007) after recognising the sequence PGRVV (Ke et al. 1997).

Fig. 2 Schematic representation of uPA. Arrow 1 shows the first cleavage between Lys158 and Ile159 to yield high molecular weight (HMW; residues 1-411) uPA. Arrow 2 shows the second cleavage between Lys135 and Ile136 to yield low molecular weight (LMW; residues 136-411) uPA and the amino-terminal fragment (ATF; residues 1-135). Created with BioRender.com.

Catalytic mechanism of uPA

Like other members of the serine protease superfamily, which comprise over one-third of all known proteolytic enzymes, (Di Cera 2009), uPA hydrolyses peptide bonds after forming a tetrahedral intermediate resulting from the nucleophilic attack of the catalytic Ser195 hydroxyl group on the carbonyl of the substrate scissile amide bond (Fig. 3) (Di Cera 2009; Hunkapiller et al. 1976). Backbone nitrogens of Gly193 and Ser195 line the oxyanion hole that stabilize the negative charge of the tetrahedral intermediate. This is followed by concerted proton transfer, hydrolysis of the intermediate and cleavage of the peptide (Di Cera 2009; Hedstrom 2002; Lee et al. 2004). The S1 pocket controls the catalytic activity (Liu et al. 2012; Spraggon et al. 1995), recognizing basic residues like Arg and Lys in substrate peptides (Rockway et al. 2002). Like many other serine proteases, experimental validation of this mechanism has been provided via mutation of Ser195 to Ala (S195A) (Alipranti et al. 2020; Gong et al. 2016) or Met (S195M) (Masih et al. 2020) to produce catalytically inactive variants.

Fig. 3 The catalytic mechanism of serine proteases. Peptide being hydrolysed is shown in black. His57, Asp189 and Ser195 constitute the catalytic triad. The backbone nitrogens of Gly193 and Ser195 comprise the oxyanion hole. uPA residues are shown in cyan.

uPA inhibitors and their binding interactions

Small molecule uPA inhibitors

As with other TLSPs, most early uPA inhibitors were arginine-mimetics, comprising an aromatic moiety substituted with an amidine (Klinghofer et al. 2001; Künzel et al. 2002; Mackman et al. 2002; Rudolph et al. 2002; Stürzebecher et al. 1999; Subasinghe et al. 2001) or guanidine (Barber et al. 2004; Fish et al. 2007; Karthikeyan et al. 2009; Sperl et al. 2000) group. These analogues were highly basic ($pK_a > 11$), positively charged at the physiological pH, showed poor pharmacokinetic properties and suffered low oral bioavailability (Rockway et al. 2002). Most inhibitors formed a salt bridge between their amidine/guanidine and the carboxylate of Asp189 located at the base of the arginine-specific S1 pocket (Spencer et al. 2002). A hydrogen bond to residue 190, contributed to selectivity for TLSPs (*e.g.* uPA, trypsin, Factor VIIa) that have Ser at this position. Other closely related TLSPs (*e.g.* tPA, thrombin, Factor Xa) have Ala at this position and lack this interaction (Katz et al. 2000). A single atom substitution that caused the displacement of the highly conserved water molecule at the S1 pocket of uPA imparted even more selectivity for Ser190 TLSPs over Ala190 relatives (Katz et al. 2001b; Mackman et al. 2001). This reasoned to be due to interactions with the side chain hydroxyl of the Ser190 that compensated for the displaced water molecule. Many inhibitors partially or fully occupied the S1 β pocket (defined by Gln192, Lys143, Ser146 and Gly219 with a disulfide Cys191-Cys220 forming its base, Fig. 4) (Wendt et al. 2004a). Inhibitor uPA selectivity was improved by increasing its occupancy of the S1 β subsite, which is absent or much smaller in closely related TLSPs (Nienaber et al. 2000b).

Fig. 4 The uPA binding site showing the S1 and S1 β pockets and their key forming residues.

Biphenyl amidine **1** is a potent and selective uPA inhibitor (IC_{50} 98 nM) but showed poor oral bioavailability likely due to the basicity of the amidine moiety (West et al. 2009). An X-ray co-crystal structure of the phenyl amidine **2** (K_i 3.8 μ M) bound to uPA (PDB 1GJA; 1.56 Å) showed the expected salt bridge between the amidine and the side chain carboxylate of Asp189 and a hydrogen bond to the carbonyl of Gly219 (Katz et al. 2001b) (Fig. 5). One of the amidine nitrogens also formed hydrogen bonds to the side chain hydroxyl of Ser190 and a conserved water molecule in the S1 pocket. The phenol group formed two hydrogen bonds with the side chain hydroxyl and backbone nitrogen of Ser195 (Spencer et al. 2002). Benzylsulfonyl-*D*-Ser-Ser-4-amidinobenzylamine **3** is a potent and selective uPA inhibitor (K_i 20 nM) that was studied in an experimental model of fibrosarcoma lung metastasis in mice (Schweinitz et al. 2004), which produced robust suppression of metastasis at relatively low dose. Its X-ray co-crystal structure with uPA (PDB 1VJA; 1.56 Å) showed the salt bridge with Asp189, hydrogen bonds between the sulfonamide and the backbone nitrogen of Gly219 and between one of the hydroxyl groups and the backbone carbonyl of Leu97B, and with the imidazole nitrogen of His99. Interactions of **3** with Leu97B and His99 appeared to impart selectivity for uPA over closely related TLSPs which have different residues at these positions (Schweinitz et al. 2004).

The X-ray co-crystal structure (PDB 1GI8; 1.75 Å) (Verner et al. 2001) of the potent uPA inhibitor 2-[2-hydroxyphenyl]-1*H*-benzimidazole-5-carboxamidine **4** (K_i 8 nM) (Katz et al. 2001a) showed a unique, multi-centered, strong hydrogen bond network involving its phenolic oxygen, benzimidazole nitrogen, a water molecule and the side chain hydroxyl of Ser195 (Verner et al. 2001). Additional interactions of this ligand with Asp189 were also mediated via a water molecule. The extensive multi-centered hydrogen bonding network prevented the ligand from fully penetrating the S1 pocket and interacting directly with Asp189 (Katz et al. 2001a). One of the amidine nitrogens formed hydrogen bonds to Gly219 while the other hydrogen bonded to the side chain hydroxyl of Ser190 and the backbone carbonyl of Val227 through a water molecule. Introduction of a chlorine atom at the 6-position of the benzimidazole ring displaced the water molecule and improved selectivity over tPA by 220-fold (Katz et al. 2001b). In uPA, the 6-chloro derivative of inhibitor **4** maintained the hydrogen bond between its amidine nitrogen and the side chain hydroxyl of Ser190, however, in the Ala190 protease tPA, this interaction was lost, explaining the marked decrease in activity for this enzyme.

4-Iodobenzo(b)thiophene-2-carboxamidine (B428) **5** is a potent uPA inhibitor (K_i 100 nM) that showed high selectivity over tPA and plasmin (Bridges et al. 1993; Klinghofer et al. 2001; Towle et al. 1993). Its X-ray co-crystal structure (PDB 1C5X; 1.75 Å) (Katz et al. 2000) showed the salt bridge between the amidine and the side chain carboxylate of Asp189, with the iodo group partially occupying the S1 β pocket (Klinghofer et al. 2001; Nienaber et al.

2000a). 2-Naphthamidine **6** was used as a starting scaffold for optimisation due to its moderate uPA inhibitory potency (K_i 5.91 μ M) and selectivity (Wendt et al. 2004b). Introduction of a phenyl amide at the 6-position yielded inhibitor **7**, which showed a $\sim$ 10-fold increase in potency (K_i 631 nM) (Wendt et al. 2004b). The X-ray co-crystal structure of **7** (PDB 1OWE; 2.0 Å) showed the salt bridge between the amidine and Asp189 as well as a hydrogen bond to the side chain hydroxyl of Ser190. The carbonyl of the phenyl amide formed a hydrogen bond with the side chain nitrogen of Gln192, while the nitrogen of the phenyl amide hydrogen bonded to the backbone oxygen of Ser214 via a water molecule (Wendt et al. 2004a; Wendt et al. 2004b). Replacement of the amide moiety with a cyclopropyl group and substitution of the phenyl ring further improved uPA activity (**8**, K_i 47 nM) and delivered oral bioavailability in rats (F_{oral} = 55%) (Bruncko et al. 2005). Introduction of substituents at the 8-position of 2-naphthamidine (inhibitors **9** K_i 40 nM, **10** K_i 30 nM and **11** K_i 0.62 nM) significantly improved uPA potency via interactions in the S1 β pocket, producing the first report of a reversible sub-nM uPA inhibitor (Klinghofer et al. 2001; Nienaber et al. 2000b; Wendt et al. 2004a). Quantum chemical calculations using density functional theory were applied to study interactions of **10** (PDB 1SQO; 1.84 Å) and **11** (PDB 1SQA; 2.0 Å) with uPA (Solis-Calero et al. 2018; Wendt et al. 2004a). Both ligands formed the salt bridge between their amidines and Asp189 and hydrogen bonds with Ser190 and Gly219. Their naphthalene cores formed hydrophobic interactions with Cys191, Gln192, Trp215 and Gly216. The aminopyrimidine at the 8-position occupied the S1 β subsite, forming hydrophobic π -sulfur type interactions with the Cys191-Cys220 disulfide. The amide nitrogen in **11** formed an additional water-mediated hydrogen bond to Ser214, its phenyl ring formed π - π stacking interactions with His57 and the terminal amino group formed a salt bridge with Asp60A (Solis-Calero et al. 2018).

In 2004, Barber *et al.* reported that 1-isoquinolinyguanidine analogue UK-356,202 **12** (K_i 37 nM) shows enhanced potency and selectivity for uPA over tPA and plasmin (Barber et al. 2004). Further evolution of the class by Pfizer led to UK-371,804 **13** (K_i 10 nM), which showed high selectivity for uPA over tPA and plasmin (Fish et al. 2007). The compound was shown to inhibit uPA in human chronic wound fluid *in vitro* (IC₅₀ 890 nM) and in a porcine acute excisional wound model *in vivo* and was selected for further preclinical evaluation as a prospective treatment for chronic dermal ulcers (Fish et al. 2007).

The clinical anticoagulant camostat **14** acts as a potent broad-spectrum inhibitor of the serine proteases trypsin, plasma kallikrein, FXIa, matriptase and uPA (IC₅₀ 87 nM) (Sun et al. 2021). The X-ray co-crystal structure of camostat **14** in complex with uPA (PDB 7DZD; 2.00 Å) showed that it inserts into the S1 pocket and is then hydrolysed to 4-

guanidinobenzoic acid (GBA), to form a covalent bond with Ser195 in addition to hydrogen bonds between the guanidine moiety and Asp189, Ser190 and Gly219.

1

IC₅₀ 98 nM

2

K_i 3.8 μM

3

K_i 20 nM

Fig. 5 Amidine and guanidine-based uPA inhibitors **1** – **14**. uPA X-ray co-crystal structures of **2**, **3**, **4**, **7**, **10**, **11** and **14** are shown highlighting the hydrogen bond interactions. Reported K_i / IC_{50} against human uPA are shown.

6-Substituted analogues of amiloride and 5-*N,N*-(hexamethylene)amiloride (HMA) as uPA inhibitors

Amiloride **15** (Midamor[®], Fig. 6) is an oral K^+ -sparing diuretic originally developed in the 1960s (Hirsh et al. 2006). The drug has been clinically used for decades in combination with hydrochlorothiazide (Moduretic[®]) or loop diuretics (*e.g.* furosemide, Frumil[®]) as an antihypertensive in patients at risk of hypokalemia during the management of hypertension, heart failure and edema (Vidt 1981). Amiloride's K^+ -sparing effect is due to selective blockade of renal epithelial sodium channels (ENaCs) in cells of the distal nephron (Warnock et al. 2014). Independent of its renal effects, amiloride has been found to have antimetastatic properties in multiple animal tumour models (reviewed extensively in (Matthews et al. 2011a)).

Amiloride **15** competitively inhibits uPA in a reversible manner (K_i 7 μ M) (Vassalli & Belin 1987) and does not affect the activity of closely related TLSPs tPA, plasmin, thrombin and kallikrein (Klinghofer et al. 2001). Its moderate potency and high selectivity indicated amiloride as an attractive scaffold for the design of improved small molecule uPA

inhibitors (Matthews et al. 2011b). Part of amiloride's anticancer effects may also be due to its inhibition of Na^+/H^+ exchanger isoforms (NHEs), particularly NHE1; which is a key regulator of intracellular pH. Accordingly, the anticancer/antimetastatic actions of amiloride perhaps arise from dual inhibition of both uPA and NHE1 (Buckley et al. 2021b; Matthews et al. 2011a). Amiloride has excellent drug properties, in part due to its unique acylguanidine group (pK_a 8.8, orally bioavailable) (Matthews et al. 2011b), unlike earlier uPA inhibitors that contain basic amidine and guanidine groups ($pK_a > 11$, Fig. 5) that contribute to poor intestinal permeability (Pajouhesh & Lenz 2005; Rockway & Giranda 2003).

In the clinic, amiloride's maximum recommended dose is limited to 20 mg/day due to the risks of hyperkalemia and cardiac arrhythmias (Sun & Sever 2020). In animal models, anticancer/metastasis effects require administration of relatively high doses of amiloride (>5 mg/kg/day), suggesting amiloride is unlikely to produce meaningful anticancer effects when given at safe doses in humans (Buckley et al. 2021a; Matthews et al. 2011a). Thus, Selective Optimisation of a Side Activity (SOSA) approach (Wermuth 2006) represented an attractive strategy to optimise amiloride into a more potent, orally active uPA inhibitor for use in cancer. Matthews *et al.* prepared several amiloride analogues and showed that the guanidine core, the 2-acylguanidine moiety and the amino groups at the 3- and 5-positions are all essential for uPA inhibitory activity, but no high potency inhibitors were identified in this study (Matthews et al. 2011b). The disruption of uPA-PAI-1 complex by amiloride and its analogues has been modelled using molecular docking simulations (Palsgaard et al. 2018).

Structure-activity relationships for 6-substituted derivatives 5-*N,N*-(hexamethylene)amiloride (HMA) **17**, a well-studied analogue that had shown similar anticancer properties, were reported by (Buckley et al. 2018). HMA **17** is also a more potent NHE1 inhibitor than amiloride **15** (Buckley et al. 2021b; Rich et al. 2000) and showed reduced diuretic effects than amiloride **15** due to weak ENaC activity (Cragoe et al. 1967). 2-Benzofuranyl analogue **16** (K_i 183 nM) demonstrated anti-metastatic properties in a mouse model of metastatic lung cancer (Buckley et al. 2019) and 4-methoxypyrimidine analogue **18** (K_i 53 nM) inhibited the formation of liver macrometastases in an orthotopic mouse pancreatic cancer model (Buckley et al. 2018). These effects were attributed to inhibition of uPA as **18** showed 143-fold selectivity for uPA over NHE1 (Buckley et al. 2021b).

The X-ray co-crystal structures of amiloride **15** (PDB 1F5L; 2.1 Å) (Zeslawska et al. 2000), HMA **17** (PDB 5ZA7; 1.7 Å) (Buckley et al. 2018) and 6-substituted analogues **16** (PDB 6AG9; 1.6 Å) (Buckley et al. 2019) and **18** (PDB 5ZAH; 2.98 Å) (Buckley et al. 2018) bound to uPA all showed the key salt bridge between the terminal nitrogens of the acylguanidine and the side chain carboxylate of Asp189 as well as hydrogen bonds to the backbone carbonyl of Gly219 and side chain hydroxyl of Ser190. The amide-like NH of the acylguanidine formed a hydrogen bond to the carbonyl of Gly219. A water-mediated hydrogen bond network is present involving a terminal nitrogen and the carbonyl atoms of the acylguanidine and the backbone amide NH and carbonyl oxygen of Val227 and amide NH of Ser214 and the terminal hydroxyl of Ser190. This water molecule is missing in the X-ray co-crystal structure of inhibitor **18** (PDB 5ZAH; 2.98 Å) (Buckley et al. 2018), however, MD simulations revealed a water molecule from the bulk enters the S1 pocket to form hydrogen bonds with Ser214 and Val227 (El Salamouni et al. 2021). The exocyclic amine at the pyrazine 3-position formed a hydrogen bond to the side chain hydroxyl of Ser195. The chlorine at the pyrazine 6-position of amiloride **15** and HMA **17** interacted with the Cys191-Cys220 disulfide and residues, Gln192, Gly216 and Gly219, which together with Lys143 and Ser146 formed the small hydrophobic S1 β subsite (Zeslawska et al. 2000). The substituents at the 6-position of the inhibitors (*e.g.* **16** and **18**) were all oriented towards the S1 β subsite. In the presence of the 4-methoxypyrimidine inhibitor **18**, the side chain of Arg217 flipped towards the S1 β pocket forming favourable interactions with the pyrimidine nitrogen (Buckley et al. 2018). Hence, increased uPA inhibitory potency of 6-substituted amiloride and HMA analogues (relative to amiloride **15** and HMA **17**) was attributed to the formation of favourable contacts between the 6-substituents and the S1 β subsite (Buckley et al. 2018; Matthews et al. 2011a). The interactions in the S1 β subsite were also thought to enhance selectivity over other TLSPs due to the absence of corresponding pockets in most related proteases (Buckley et al. 2018).

Fig. 6 uPA inhibitors amiloride **15**, HMA **17** and 6-substituted analogues **16** and **18** and their uPA X-ray co-crystal structures showing key hydrogen bond interactions. Reported K_i / IC_{50} against human uPA are shown.

Non-amidine and guanidine-based uPA inhibitors

There have been several efforts to design potent, selective and orally bioavailable uPA inhibitors that substitute amidine and guanidine moieties of uPA inhibitors with less basic functional groups that maintain key interactions with Asp189 (Venkatraj et al. 2012). Tranexamic acid (TXA) **19** is an antifibrinolytic (McCormack 2012) and weak active site inhibitor of uPA (K_i 2 mM, Fig. 7) (Wu et al. 2019). Its uPA X-ray co-crystal structure (PDB 6NMB; 2.30 Å) showed that the compound binds in the S1 pocket and forms hydrogen bonds with the side chain carbonyl of Asp189, the backbone carbonyl of Ser190 and side chain hydroxyl of Ser195. Hydrophobic interactions were formed between the cyclohexane ring and two cysteines (Cys191 and Cys219) as well as with Gly216 (Wu et al. 2019). YO-2 **20** (IC_{50} 3.99 μ M) and PSI-112 **21** (IC_{50} > 25 μ M) are derivatives of TXA **19** that inhibited uPA in the micromolar range (Law et al. 2017; Tsuda et al. 2021). YO-2 **20** is more active than PSI-112 **21** due to interaction of Tyr60 in uPA with the pyridine ring of YO-2 **20**, while it seemed to sterically clash with the quinoline moiety in PSI-112 **21** together with the side chain of Arg35. PSI-112 is more selective for plasmin over uPA with Phe587 acting as a key residue in plasmin for the binding of these inhibitors. Loss of interactions with the equivalent residue Val41 in uPA was thought to explain this selectivity (Law et al. 2017).

Optimization of earlier inhibitor **1** (Fig. 5) led to **22** (PDB 3IG6; 1.83 Å), a more potent (IC_{50} 25 nM, Fig. 7) and selective inhibitor of uPA that maintained the salt bridge with Asp189 via a primary amino group (West et al. 2009). The amine also formed hydrogen bonds with the backbone carbonyls of Ser190 and Gly218. The carboxylic acid moiety formed hydrogen bonds to the side chain imidazole and hydroxyl of the catalytic His57 and Ser195, respectively, as well as the backbone nitrogen of Gly193. Despite this, the compound showed no oral bioavailability due to the additional basic dimethylamino pyrrolidine. However, the truncated derivative ZK824859 **23** (IC_{50} 79 nM) showed acceptable oral bioavailability in rats (F_{oral} = 30%) (Islam et al. 2018). A model structure of compound **23** based on the X-ray co-crystal structure of **22** showed that it maintains the salt bridge interaction with Asp189, forms hydrogen bonds between its primary amine and the backbone carbonyls of Ser190 and Gly218 and between its carboxylic acid and the side chain imidazole of His57, backbone nitrogen of Gly193 and the side chain hydroxyl of Ser195 (Islam et al. 2018). Selectivity of ZK824859 **23** for uPA over tPA was attributed to the positioning of the difluoropyridine ring close to His99, as residue 99 in tPA is Tyr, which could sterically clash with a fluorine atom (Islam et al. 2018). 2-Amino-5-hydroxybenzimidazole **24** (pK_a 7.4) showed moderate activity against uPA (IC_{50} 10 μ M) (Hajduk et al. 2000) and its uPA X-ray co-crystal structure (PDB 1FV9; 3.00 Å) showed a salt bridge between the 2-amino group and Asp189 and hydrogen bonds between the imidazole and Gly218. Replacing the amide linker of naphthamidine inhibitor **11** (Fig. 5) with a sulfonamide and the aryl amidine with a methyl ester yielded **25** (Fig. 7), which retained some uPA potency (K_i 2.8 μ M) and maintained selectivity over other TLSPs (Venkatraj et al. 2012).

4-Bromobenzylamine **26** is a weak inhibitor of uPA (K_i 1.28 mM), however, its uPA X-ray co-crystal (PDB 5YC6; 1.18 Å) revealed that this simple compound buries deeply into the S1 pocket and forms a non-covalent halogen bond between the electron-withdrawing bromine atom and the electron-rich Asp189. The bromine atom also made two interactions with the backbone carbonyl and side chain hydroxyl of Ser190, and van der Waals interactions were seen between its phenyl group and Ser190, Cys191, Gln192, Trp215, Gly216 and Arg217 (Jiang et al. 2018).

Fig. 7 Non-amidine and guanidine-based uPA inhibitors **19** – **26**. X-ray co-crystal structures of **19**, **22**, **24** and **26** showing key binding interactions. Reported K_i / IC_{50} against human uPA are shown.

Clinical studies with uPA inhibitors

The first and most advanced uPA inhibitor in oncology studies was the oral small molecule antimetastatic and non-cytotoxic 3-amidinophenylalanine WX-UK1 (Mesupron®) **27** and its hydroxyamidine prodrug WX-671 **28** (Fig. 8), both developed by WILEX AG, Germany (Fathi et al. 2019). Mesupron® **27** successfully completed several phase I (Goldstein 2008) and II clinical trials in pancreatic and breast cancer. In 2014, RedHill Biopharma acquired the exclusive worldwide development and commercialization rights to Mesupron® **27** for all indications (Banyś-Paluchowski et al. 2019). Mesupron® **27** is a modest uPA inhibitor (K_i 410 nM) (Stürzebecher et al. 1999) but is non-selective and inhibits closely related TLSPs (Setyono-Han et al. 2005; Xu et al. 2017). The amidine moiety of Mesupron® prodrug WX-671 **28** inserts into the S1 pocket of uPA to form the salt bridge with Asp189 with the rest of molecule interacting with residues in 216-218 and 99 loops (Fathi et al. 2019). It was recently found that WX-UK1 **27** is a nanomolar inhibitor of the human serine proteases trypsin-6, trypsin-3, trypsin-2, trypsin-1 and matriptase-1 (Oldenburg et al. 2018). As such, it is unclear to what

extent the clinical efficacy reported for Mesupron[®] is attributable to uPA inhibition over its more potent effects on other cancer-relevant proteases.

27
WX-UK1
 K_i 410 nM
28 R = OH
WX-671

Fig. 8 Clinical uPA inhibitors WX-UK1 (Mesupron[®]) **27** and its hydroxyamidine prodrug WX-671 **28**. Reported K_i against human uPA are shown.

Ligand-based design of uPA inhibitors

Using seven diverse sets of inhibitors, the pharmacophoric space of 202 uPA inhibitors was explored and three high quality models were developed showing binding interactions comparable to those seen in the crystal structures of inhibitors bound to uPA (Al-Sha'er et al. 2014). This was followed by quantitative structure activity relationship (QSAR) analysis, where using the pharmacophoric models and QSAR equation to screen the National Cancer Institute (NCI) libraries identified four hits **29** – **32** that showed moderate potency against uPA (IC_{50} 6.3 – 28.4 μ M, Fig. 9).

Fragment-based discovery of uPA inhibitors

Fragment based drug discovery (FBDD) uses low molecular weight compounds (fragments), selected based on their ability to bind to the target as starting points for hit to lead optimization (Denis et al. 2021; Giordanetto et al. 2019). Using FBDD, the oral antiarrhythmic drug, mexiletine **33** (IC_{50} > 1mM), a fragment hit from X-ray crystallographic screening against uPA, was selected as a starting fragment for optimisation. The program delivered compound **34** as a potent (IC_{50} 72 nM), selective, weakly basic (pK_a 8.7) and orally bioavailable (F_{oral} = 60%) uPA inhibitor (Frederickson et al. 2008). While the X-ray co-crystal structure of mexiletine **33** (PDB 2VIN; 1.90 Å) did show the salt bridge between its amine and Asp189, as well as hydrogen bonds to the backbone carbonyls of Ser190 and Gly219, these interactions were weaker with the more potent derivative **34** (PDB 2VIW; 2.05 Å). Instead, water-mediated hydrogen bonds were formed between

the amide carbonyl of **34** and the side chain imidazole nitrogen of His99 and the backbone carbonyl of Ser214. Amide substituted imidazo[1,2-a]pyridine analogues *e.g.* **35** that showed high uPA potency (IC_{50} 97 nM) and greater than 1000-fold selectivity over other TLSPs were developed from an imidazopyridine starting scaffold (Gladysz et al. 2015). Docking of these analogues showed the common hydrogen bonds with Asp189, Ser190, Gly219 and an additional hydrogen bond between the amide NH and Tyr151.

29
NCI0135766
 IC_{50} 6.3 μ M

30
NCI0666712
 IC_{50} 9.0 μ M

31
NCI004367
 IC_{50} 11.3 μ M

32
NCI0144205
 IC_{50} 28.4 μ M

33
Mexiletine
 IC_{50} > 1 mM

34
 IC_{50} 72 nM

35
 IC_{50} 97 nM

Fig. 9 uPA inhibitors **29** – **32** identified through screening of NCI libraries, and compounds **33** – **35** from fragment-based screening programs. The uPA X-ray co-crystal structure of **34** showing key hydrogen bond interactions. Reported K_i / IC_{50} against human uPA are shown.

Structure-based design of uPA inhibitors

Until the mid-1990s, no crystallographic information existed on full length, single or two chain uPA, precluding structure-based drug design (SBDD) programs (Spraggon et al. 1995; Zeslawska et al. 2000). In 1995, Spraggon *et al.* reported the first X-ray co-crystal structure of the catalytic domain of human uPA complexed with the irreversible inhibitor Glu-Gly-Arg chloromethyl ketone (EGR-cmk **36**, Fig. 10). The structure revealed that the enzyme has an S1 specificity pocket similar to trypsin, a less accessible hydrophobic S2 pocket and a solvent-accessible S3 pocket capable of accommodating a wide range of amino acids (Spraggon et al. 1995). The arginine moiety in EGR-cmk **36** bound to Asp189 in the S1 pocket, with one of the guanidine nitrogens hydrogen bonding to the side chain hydroxyl of Ser190 and the backbone carbonyl of Gly218. A salt bridge and a hydrogen bond were formed between the glutamate in EGR-cmk **36** and Arg217 and Glu218, respectively (Spraggon et al. 1995).

In 2000, Zeslawska *et al.* reported the X-ray co-crystal structure of a novel truncated variant of human uPA in complex with amidinophenylalanine derivative UKI-1D **37** (PDB 1F92, 2.6 Å, K_i 640 nM) (Zeslawska et al. 2000). This structure showed that the amidine buried deeply into the S1 pocket to form the salt bridge with Asp189. The amidine also formed hydrogen bonds with the backbone carbonyls of Ser190 and Gly219. The alanine nitrogen was directed away from His99 and a hydrogen bond was present between the nitrogen of the terminal amine group and Tyr94. Hydrophobic van der Waals interactions were formed with His57, Leu97B, His99 and Trp215.

In the same year, Sperl *et al.* reported the X-ray co-crystal structure of human uPA complexed with *N*-(1-adamantyl)-*N'*-(4-guanidinobenzyl)urea WX-293T **38** (PDB 1EJN; 1.8 Å) (K_i 2.4 μM) (Sperl et al. 2000). The phenylguanidine moiety inserted into the S1 specificity pocket and formed the salt bridge with Asp189. Hydrogen bonds were formed with Ser190, Gly193, Ser195 and Gly219. The ureido group made new interactions with the hydrophobic S1' subsite, comprising the Cys58-Cys42 disulfide and the backbone carbonyls of Val41 and His57 (Sperl et al. 2000; Zeslawska et al. 2000).

Molecular docking of uPA inhibitor UK122 **39** (K_i 640 nM) showed the phenylamidine moiety forming the salt bridge to Asp189 and hydrogen bonds to Ser190 in the S1 pocket (Zhu et al. 2007). The oxazolidinone moiety formed hydrogen bonds with His99, Asp194, Ser195 and Gly198. UK122 **39** inhibited migration and invasion of CFPAC-1 pancreatic cancer cells *in vitro* (Zhu et al. 2007).

In 2014, Sa *et al.* studied the uPA binding modes of five 1-(7-sulfonamidoisoquinoliny)guanidine inhibitors **40** – **44** using binding free energy calculations with the Molecular Mechanics / Poisson–Boltzmann Surface Area (MM/PBSA) method (Sa et al. 2014). The calculated free energies were consistent with earlier experimentally-determined values (Fish et al. 2007). For the five uPA inhibitor complexes, a Molecular Mechanics / Generalized Born Surface Area (MM/GBSA) free energy decomposition analysis revealed that Asp189 makes the most favourable contribution to the binding free energy, followed by Ser190, Cys191 and Gln192, along with Trp215, Gly216, Arg 217, Gly218 and Cys 219 (Sa et al. 2014).

Fig. 10 Chemical structures of inhibitors **36** – **44** and X-ray co-crystal structures of **37** and **38** showing key interactions with uPA. Reported K_i against human uPA are shown.

Cyclic peptide uPA inhibitors

The design of peptides that target serine proteases has attracted much interest (Li et al. 2019) and several disulfide-bridged cyclic peptide uPA inhibitors have been developed (Fig. 11). Upain-1 **45** (CSWRGLENHRMC, K_i 500 nM) is a highly specific competitive inhibitor of human uPA (Hansen et al. 2005). An X-ray co-crystal structure of upain-1 **45** bound to uPA (PDB 2NWN, 2.15 Å) showed direct interactions with Arg35, His57, Asp60A, Tyr64, His99, Asp189, Ser190, Gln192, Gly193, Ser195, Gly216 and Gly219. A pair of water-mediated interactions were present between two conserved water molecules and residues Gly216, Gly219, Ile60 and Asp60A (Zhao et al. 2007). Upain-1 **45** also extensively interacted with residues comprising 37- and 60-loops as well as His99. These residues are significantly different in human and mouse uPA and in related TLSPs (Zhao et al. 2007), which imparted selectivity of upain-1 **45** for human uPA. Alanine scanning of residues Asp57, Asp60A, Tyr94, His99, Tyr151, Trp186, Asp189 and Trp215 produced 10-fold reductions in binding affinity (Hansen et al. 2005). Upain-1-W3F mutant **46** (CSFRGLENHRMC, PDB 6XVD 1.40 Å) was found to be a weaker uPA inhibitor (K_i 149 μM) (Xue et al. 2020).

The bicyclic peptide **47** (ACSRYEVDCRGRGSACG) is a potent (K_i 53 nM) and selective inhibitor of uPA (Angelini et al. 2012). The presence of two small, constrained loops rather than a single loop proffered multiple improved interactions with uPA. The uPA X-ray co-crystal structure of **47** (PDB 3QN7, 1.90 Å) showed hydrogen bonds with His57, Asp60A, Tyr60B, Val141, Gln192, Gly193, Asp189, Ser190, Ser195 and Gly218. Close contacts (distances < 4 Å) with Arg35, His37, Arg37A, Thr39, Gly37B, His99, Tyr151, Asp194, Lys224, Pro225 and Gly226 were also observed.

With an absence of specific inhibitors of mouse uPA for use in mouse cancer models, cyclic peptide inhibitor mupain-1 **48** (CPAYSRYLDC, K_i 500 nM) was designed to target mouse uPA (Andersen et al. 2008; Zhao et al. 2014). A three-dimensional model of mupain-1 **48** at the binding site of mouse uPA showed interactions with Lys41, His57, Tyr99, Ser195, Asp189 and Lys192 (Andersen et al. 2008). His99 in human uPA is important for imparting selectivity of upain-1 **45** and Tyr99 in mouse uPA for mupain-1 **48** (Zhao et al. 2007). Substituting His99 in human uPA to Tyr99 (as in mouse uPA), led to a 100-fold increase in affinity for human uPA (Andersen et al. 2008). The X-ray co-crystal structure of the partially murinised human uPA (H99Y) in complex to mupain-1 **48** (PDB 4X1Q, 2.28 Å) revealed interactions with Arg35, Cys58, Thr97A, Leu97B, Tyr99, Asp189, Ser190, Gln192, Gly193, Arg217 and Gly219 (Zhao et al. 2014). In general, inhibitor binding to uPA resulted in entropic loss, hence increasing the rigidity of the inhibitors improved entropic contributions to binding (Roodbeen et al. 2013; Zhao et al. 2014). In contrast to upain-1 **45** and other serine protease inhibitors, increasing the flexibility of mupain-1 **48** improved binding affinity for uPA (Xu et al. 2017) due to more favourable exosite interactions (Zhao et al. 2014).

45
Upain-1
 K_i 500 nM

47
 K_i 53 nM

48
 Mupain-1
 K_i 500 nM

Fig. 11 X-ray co-crystal structures of cyclic peptide uPA inhibitors **45**, **47** and **48** showing key interactions. Reported K_i against human uPA for **45** and **47** and against the partially murinised human uPA (H99Y) for **48** are shown.

Species specificity of uPA inhibitors

Studies have shown that uPA secreted by the tumour stroma plays an important role in cell growth and dissemination in xenograft rodent models (Frandsen et al. 2001; Rømer et al. 1994). Some studies suggested that tumour stromal cells (*e.g.* endothelial cells, fibroblasts and macrophages) are in fact the predominant uPA expressing tissue (Hildenbrand et al. 2009). Tumour and stromal expression of uPA and/or uPAR at the invasive front of tumours has been demonstrated in human breast xenograft models (Frandsen et al. 2001; Ploug et al. 2001; Rømer et al. 1994) and in patient specimens (Alpízar-Alpízar et al. 2012; Brungs et al. 2017; Illemann et al. 2009; Pyke et al. 1991). Anticancer therapies should therefore target tumour and stromal uPA as they both play an important role in tumour invasion and proliferation (Hofmeister et al. 2008). This creates problems, however, when inhibitors show differences in potency against uPA from human tumour xenografts and uPA secreted by the mouse-derived stromal tissues as it may confound interpretation of on-target efficacy.

Human and mouse uPA are highly homologous, with their protease catalytic domains showing an overall identity of 71% (Klinghofer et al. 2001). Their active sites contain only four different residues: Asp60, His99, Ser146 and Gln192 in human uPA and Gln60, Tyr99, Glu146 and Lys192 in mouse uPA (Klinghofer et al. 2001) (Fig. 12). Despite this relatively small difference, many small molecule uPA inhibitors show higher potency against human than mouse uPA (Buckley et al. 2018; Klinghofer et al. 2001).

Fig. 12 Superimposed X-ray co-crystal structures of human (PDB 1F5L; 2.1 Å) (Zeslowska et al. 2000) and mouse (PDB 5LHQ) (Kromann-Hansen et al. 2017) uPA illustrating the four residues (60, 99, 146 and 192) that are close to their binding sites and different between both species.

In 2001, Klinghofer *et al.* studied the human/mouse species differences for a number of amidine-based uPA inhibitors (Klinghofer et al. 2001). From the X-ray co-crystal structures of B428 **5** (human uPA K_i 100 nM and mouse uPA K_i 82 nM), 2-naphthamidine **6** (human uPA K_i 5.9 μ M and mouse uPA K_i 6.0 μ M) and amiloride **14** (human uPA K_i 2.8 μ M and mouse uPA K_i 1.7 μ M), they concluded that these inhibitors occupy only the S1 pocket, which has similar residues in both species. Accordingly, these inhibitors didn't show significant species specificity (i.e. human/mouse selectivity ratios $\sim$ 1) (Buckley et al. 2018; Klinghofer et al. 2001). Amiloride **14** and B428 **5** bear a halogen substituent that can access part of the S1 β pocket and show higher inhibitory potency than 2-naphthamidine **6** (Nienaber et al. 2000b). Thus, the S1 β pocket was targeted in SBDD efforts to create more potent uPA inhibitors. Naphthamidine analogues with larger structural extensions displayed higher potency for human over mouse uPA (i.e. increased human/mouse selectivity ratios). Among this series, the most potent analogue against human uPA was the disubstituted naphthamidine **11** (human uPA K_i 0.64 nM and mouse uPA K_i 130 nM), which showed human/mouse selectivity ratio of 203 (Klinghofer et al. 2001). With groups extending beyond the S1 pocket and achieving potency gains by making interactions with the four residues that differ between human and mouse uPA, this compound showed a pronounced selectivity for human over mouse uPA (Klinghofer et al. 2001).

Species specificity of HMA and 6-substituted analogues

While amiloride **14** inhibited human and mouse uPA almost equally (Buckley et al. 2018; Klinghofer et al. 2001), HMA **16**, showed higher selectivity for human over mouse uPA (human uPA K_i 1,356 nM, mouse uPA K_i 9,308 nM, human/mouse selectivity ratio: 6.9). Selectivity was further increased in HMA analogues obtained by introduction of substituents at its 6-position (*e.g.* compound **15**, human uPA K_i 53 nM, mouse uPA K_i 1,611 nM, human/mouse selectivity ratio: 30.4). With some other derivatives, selectivity for the human enzyme was around 130-fold higher than mouse (Buckley et al. 2018). Such large species differences may complicate development of these inhibitors due to poor inhibition of mouse uPA affecting interpretation of data from human-mouse xenograft tumour models (Schweinitz et al. 2004). Inhibitors effective against both enzymes would offer greater confidence that observed efficacy arises from uPA inhibition, particularly for the determination of minimal effective dosing. Human over mouse species selectivity observed with the 6-substituted HMA analogue **15** was decreased for the corresponding amiloride analogue **49** (human uPA K_i 204 nM, mouse uPA K_i 956 nM, human/mouse selectivity ratio: 4.7, Fig. 13). While both inhibitors showed similar interactions with human uPA, the disruption of the water network in presence of mouse uPA was responsible for the observed species selectivity. In **15**, the 5-*N,N*-hexamethylene ring caused unfavourable steric expulsion of a water molecule in presence of mouse uPA (residue 99 is Tyr), while this water molecule was maintained in presence of human uPA (residue 99 is His). In contrast amiloride analogue **49** with its smaller 5-NH₂ group maintained similar water networks when bound to both human and mouse uPA and didn't display pronounced species selectivity (El Salamouni et al. 2021).

15 bound to human uPA

$K_i = 53$ nM

49 bound to human uPA

$K_i = 204$ nM

15 bound to mouse uPA

$K_i = 1,611$ nM

49 bound to mouse uPA

$K_i = 956$ nM

Human/Mouse selectivity factor = 30.4

Human/Mouse selectivity factor = 4.7

Fig. 13 Differences in water networks and changes at residue 99 explain the human and mouse species differences for inhibitors **15** and **49**. Reported K_i against human (top) and mouse (bottom) uPA are shown.

Conclusion

uPA is an important anti-metastasis drug target that has attracted ongoing efforts to develop potent and selective inhibitors with good drug properties. While the earlier highly basic amidine and guanidine-based inhibitors showed poor oral bioavailability, potent less basic inhibitors of uPA have been developed. 6-Substituted amiloride and HMA analogues that show nanomolar uPA potency and high selectivity over other TLSPs were recently described. The availability of X-ray co-crystal structures of these inhibitors in complex with uPA has enabled an intricate understanding of the key binding interactions. Most inhibitors maintain the critical salt bridge with Asp189 in the S1 pocket. Interactions of some inhibitors with Ser190 confer selectivity for uPA over closely related TLSPs that instead have Ala190. Many uPA inhibitors form

hydrogen bonds with Gly219 located at the periphery of the S1 β subsite, with those extending deeper showing high affinity. While aiming to develop inhibitors that can target both human and mouse uPA to be used reliably in cancer models, some analogues show human/mouse species selectivity, which may complicate the interpretation of efficacy signals from xenografted mouse models of cancer. Recent computational findings supported by experimental data suggested that 6-substituted amiloride analogues are less likely to show human/mouse species selectivity and that residue 99 is a key contributor to this observed selectivity.

Conflict of interest

The authors declare no conflicts of interest.

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