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Characterising the Subsite Specificity of Urokinase-Type Plasminogen Activator and Tissue-Type Plasminogen Activator using a Sequence-Defined Peptide Aldehyde Library

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Urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) are two serine proteases that contribute to initiating fibrinolysis by activating plasminogen. uPA is also an important tumour-associated protease due to its role in extracellular matrix remodelling. Overexpression of uPA has been identified in several different cancers and uPA inhibition has been reported as a promising therapeutic strategy. Although several peptide-based uPA inhibitors have been developed, the extent to which uPA tolerates different tetrapeptide sequences that span the P1–P4 positions remains to be thoroughly explored. In this study, we screened a sequence-defined peptide aldehyde library against uPA and tPA. Preferred sequences from the library screen yielded potent inhibitors for uPA, led by Ac-GTAR-H (K_i = 18 nM), but not for tPA. Additionally, synthetic peptide substrates corresponding to preferred inhibitor sequences were cleaved with high catalytic efficiency by uPA but not by tPA. These findings provide new insights into the binding specificity of uPA and tPA and the relative activity of tetrapeptide inhibitors and substrates against these enzymes.

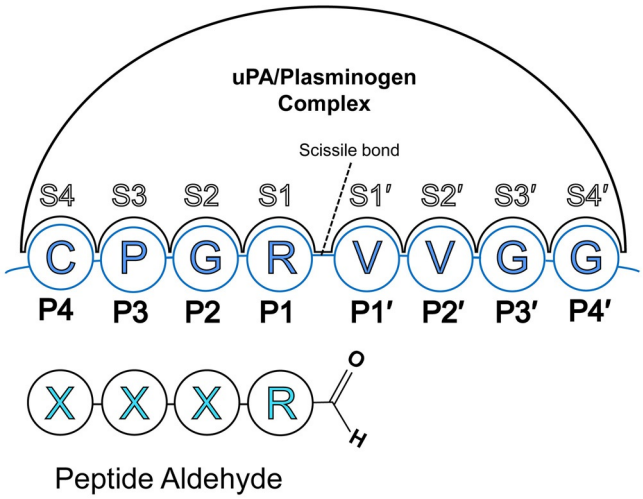

Figure 1. Schematic diagram showing binding interactions between uPA and plasminogen or a peptide aldehyde inhibitor. The substrate binding sites (S4–S4′) and protease binding residues (P4–P4′) are named according to Schechter and Berger nomenclature. X represents a variable residue.

The plasminogen activation system is an important proteolytic pathway that has diverse biological functions, including fibrinolysis and tissue remodelling.[1,2] The core components of the system are three serine proteases, namely plasminogen and its endogenous activators: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasminogen is a protease zymogen that is activated by cleavage of the Arg561-Val562 peptide bond (Figure 1) to yield plasmin, a key enzyme that has several functions in extracellular matrix (ECM) remodelling. These functions include cleaving a range of ECM proteins and activating additional proteases, such as matrix metalloproteases.[3] Plasminogen activation can be carried out by either tPA or uPA and each activator has been proposed to have different temporal and spatial functions. tPA binds strongly to fibrin and is regarded to be primarily involved in activating rapid fibrinolysis.[4,5] By contrast, uPA is considered to be important for tissue remodelling as binding to its cell-surface receptor (uPAR) provides a mechanism for precise directional control of fibrinolysis and ECM remodelling, for example to guide cell migration.[6] However, these functions are not mutually exclusive. Gene knockout studies in mice have identified a degree of redundancy between tPA and uPA as loss of one plasminogen activator does not cause significant phenotypic effects.[7]

ECM remodelling is widely recognised as a critical step in cancer progression and metastasis. Accordingly, dysregulation of uPA and the plasminogen activation system has been reported in several different cancers, including breast cancer, liver cancer and pancreatic cancer.[8] The link between uPA and cancer has been most clearly established in breast cancer, the most common malignancy in women and the second leading cause of cancer deaths.[9] uPA has been validated as a prognostic biomarker for breast cancer in separate clinical studies, with higher tumour levels of uPA correlating with poor patient prognosis.[10,11] Additionally, in liver and pancreatic cancer,

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higher expression of uPA has been reported to contribute to tumour invasion and metastasis, resulting in poor survival.[12,3] Other malignancies that have been reported to show elevated expression of uPA include prostate, ovarian, cervical, endometrial, colorectal, gastric, oral, oesophageal and bladder cancer.[14] In addition to being a valuable biomarker, several studies have demonstrated that uPA is a therapeutic target for different cancers by focusing on either the expression, activation or proteolytic activity of uPA. In a transgenic breast cancer model, uPA-deficient mice showed markedly lower levels of tumour metastasis compared to wild-type animals,[15] whereas gene knockdown of uPA or uPAR by RNA interference was shown to inhibit tumour invasion in breast cancer and prostate cancer models.[16,17] Similarly, uPA inhibitors, including antibodies or peptidomimetics, have been shown to block tumour invasion and metastasis in fibrosarcoma models.[18,19] Accordingly, there has been long-standing interest in developing inhibitors for uPA as a strategy to limit tumour progression and metastasis in several cancers.

Different types of uPA inhibitors have been developed including small molecules, peptides and proteins. A variety of peptide-based uPA inhibitors have been engineered, which can be classified into two categories: linear peptide inhibitors (typically comprising three or four amino acids) and cyclic peptide inhibitors.[20–23] Several tripeptide inhibitors have been reported to inhibit uPA with inhibition constants in the nanomolar range.[20,21,24] These inhibitors are based on a similar tripeptide sequence (D-SAR) and were developed by optimising the inhibitor's C-terminal warhead or by modifying the N-terminal functional group. For example, the peptide aldehyde inhibitors (for example, $BnSO_2$-D-SAR-H) and 4-amidinobenzylamine-based peptide inhibitors (for example, benzylsulfonyl-D-SA-4-amidinobenzylamide) have similar P3–P1 sequences.[20,21] The cyclic peptide inhibitors were developed using a different strategy that involved screening uPA against sequence-diverse libraries using phage display.[22,25] The most potent cyclic peptide inhibited uPA with a K_i of 53 nM, and was further optimised by substituting a Gly residue in the binding loop with various D-amino acids.[25–26] Since uPA tolerates binding loop substitutions in cyclic peptide inhibitors, we hypothesised that, for linear peptide inhibitors, sequences other than D-SAR might also produce potent uPA inhibitors. However, the extent to which uPA tolerates different sequences in linear peptide inhibitors has yet to be thoroughly explored.

Although specificity data for linear peptide uPA inhibitors is relatively limited, the substrate specificity of uPA has been examined in detail using a positional scanning substrate combinatorial library and phage display.[27–29] In studies with other serine proteases, including several kallikrein-related peptidases, cathepsin G and plasmin,[30–33] we used the specificity data from positional scanning to design a non-combinatorial substrate library (sparse matrix library) to identify preferred substrate sequences for each enzyme. In the present study, we used an alternative approach and synthesised a non-combinatorial library of peptide aldehyde inhibitors (Figure 1). The sequences included in the peptide aldehyde library were based on the preferred residues at each subsite for uPA and tPA identified by positional scanning.[27] Both uPA and tPA have trypsin-like specificity and prefer Arg over Lys, thus Arg with a C-terminal aldehyde group (abbreviation: −H) was selected as the P1 residue (Table 1). uPA and tPA prefer similar residues at the P2 position, with the three most preferred residues being Ala, Ser and Gly. However, each protease favours different residues at P3. Therefore, we selected the two most preferred residues for uPA (Thr and Ser) and for tPA (Phe and Tyr) at P3. uPA and tPA also prefer different amino acids at P4 and four residues were selected for the inhibitor library: Gly and Asn (preferred by uPA), together with Phe and Gln (preferred by tPA). The resulting 48 peptide aldehydes were produced by solid-phase synthesis using Fmoc chemistry.

Screening uPA against the peptide library at a concentration of 0.1 μM revealed that peptide aldehydes with P2 Ala and Gly typically inhibited uPA at similar levels, while those with P2 Ser were much less potent (Figure 2). Among the peptide aldehydes with the same P2 residue, inhibitors with P3 Thr generally displayed stronger activity than those with P3 Ser, Tyr or Phe. The four most potent uPA inhibitors were Ac-GTGR-H, Ac-GTAR-H, Ac-QTGR-H and Ac-FTAR-H. These four compounds have the same P3 residue, but different residues at P2 and P4. Two of them contain P2 Ala, which is present in many of the reported tripeptide inhibitors (eg. $BnSO_2$-D-SAR-H), whereas the other two have P2 Gly. At P4, both small residues (Gly) and large residues (Gln or Phe) were present.

The most preferred inhibitors from the library screen were subsequently purified and assays were performed to determine the inhibition constant (K_i). The results showed that the most potent compound was Ac-GTAR-H ($K_i = 18(\pm 1)$ nM, Table 2), followed by Ac-GTGR-H (twofold less potent) and Ac-FTAR-H (fourfold less potent). However, the inhibitor with the highest activity in the library screen appeared to be Ac-GTGR-H. This result may be due to the fact that in the library screen, the activity of each peptide was determined using a single concentration of inhibitor (100 nM), whereas a range of inhibitor concentrations were tested in the assays used to determine K_i. Interestingly, previous studies have shown that peptide aldehydes with an N-terminal benzoyl (Bz) group in the P4 position

Table 1. Design of the peptide aldehyde library.

Method	P4	P3	P2	P1
Positional scanning library (uPA)[27]	G > N > n[a] > F	T > S > Y > A	A > S > G	R
Positional scanning library (tPA)[27]	F > Q > W > N	F > Y > I > E	S > G > A	R
Peptide aldehyde library	G, N, F, Q	T, S, F, Y	A, S, G	R

[a] n = norleucine.

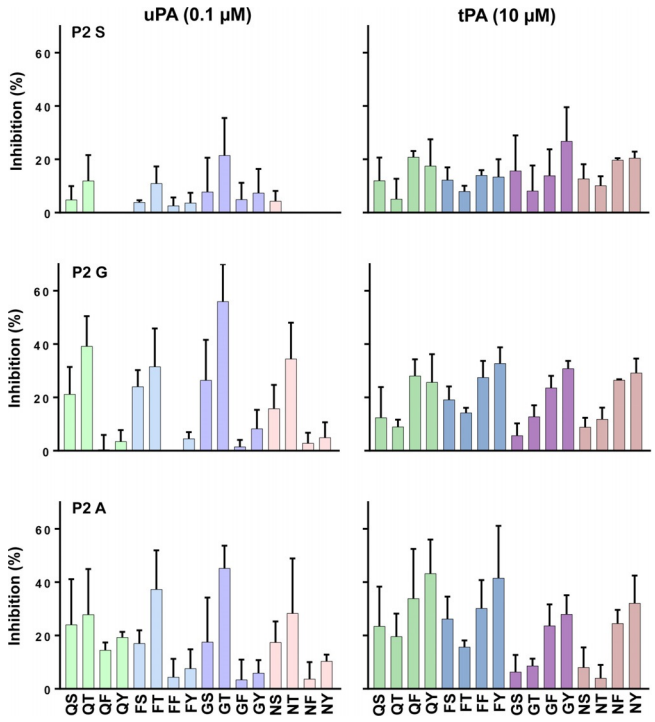

Figure 2. Screening the P1–P4 specificity of uPA and tPA using a peptide aldehyde library. Data is expressed as mean ± SD from three independent experiments. The P4–P3 sequences are given across the *x*-axis, the P2 residue is given on the *y*-axis and the P1 residue is fixed (Arg). The % inhibition (*y*-axis) for each variant was calculated by comparing kinetic rates to control assays without inhibitor.

Table 2. Inhibition constants for peptide aldehyde inhibitors with uPA.

Compound	Sequence	K_i [nM] ± SEM
1	Ac-GTGR-H	42 ± 2
2	Ac-GTAR-H	18 ± 1
3	Ac-FTAR-H	72 ± 6
4	Bz-TAR-H	51 ± 1

are potent uPA inhibitors[20] and we found that Ac-FTAR-H was among the most preferred sequences. Accordingly, we performed an additional substitution where P4 Phe was replaced with an N-terminal Bz group to generate a new compound, Bz-TAR-H. This modification produced a 1.4-fold increase in potency compared to Ac-FTAR-H (Table 2). However, Bz-TAR-H was less potent than Ac-GTAR-H and Ac-GTGR-H. Compared to linear peptide inhibitors reported in previous studies, the activity of Ac-GTAR-H is in a similar range to peptide aldehydes based on the D-SAR sequence.[20] However, our compound contains Thr and Gly at the P3 and P4 positions, respectively, whereas the existing inhibitors have D-Ser at P3 and an aryl sulfonamide group at P4.

Peptide aldehydes are classified as substrate analogues and their mode of action involves enzyme-mediated production of a hemiacetal species that mimics the transition state formed during typical substrate catalysis.[34] To explore whether the inhibitor sequences were also preferred substrates sequences, peptide *para*-nitroanilide (pNA) substrates were synthesised and assayed against uPA. As shown in Table 3, the highest catalytic efficiency was provided by the substrate containing the sequence from the most potent inhibitor (Ac-GTAR-H). Three of the peptide-pNA substrates showed similar K_M and k_{cat} values, whereas Ac-GTGR-pNA displayed higher K_M and k_{cat} values. When compared to the most commonly used uPA substrate (Pyr-Gly-Arg-pNA),[35,36] the catalytic efficiency for Ac-GTAR-pNA was in a similar range ($1 \times 10^5\ M^{-1} s^{-1}$), but the K_M value was approximately fourfold lower.

Next, we screened the peptide aldehyde library against tPA. Overall, the level of inhibition against tPA was much lower than for uPA and the library was screened at 10 μM. Peptide aldehydes with P2 Ala and Gly were slightly more potent against tPA than P2 Ser, whereas aromatic residues (Phe and Tyr) were preferred at P3 (Figure 2). The two most potent tPA inhibitors were Ac-QYAR-H and Ac-FYAR-H. Additionally, the most potent peptide aldehydes for uPA (Ac-GTGR-H and Ac-GTAR-H) displayed much weaker activity against tPA, indicating that these two peptide aldehydes were selective for uPA over tPA. Consistent with this, peptide aldehyde inhibitors based on the D-SAR sequence have been reported to display more than 100-fold selectivity for uPA over tPA as they show weak activity against tPA.[21]

To investigate the weak inhibition of peptide aldehydes against tPA, we synthesised two additional peptide-pNA substrates based on Ac-QYAR-H and Ac-FTAR-H. These two substrates, together with the four uPA peptide substrates, were assayed towards tPA. The results showed that the highest catalytic efficiency towards tPA was given by the substrate corresponding to the most potent inhibitor (Ac-QYAR-H). However,

Table 3. Michaelis–Menten kinetic constants for peptide-pNA substrates with uPA and tPA.

Compound	Protease	K_M [μM] ± SEM	k_{cat} [s^{-1}] ± SEM	k_{cat}/K_M [$M^{-1} s^{-1}$] ± SEM
Ac-GTGR-pNA	uPA	62 ± 4.0	4.58 ± 0.12	74 300 ± 5100
	tPA	760 ± 190	0.21 ± 0.03	280 ± 81
Ac-GTAR-pNA	uPA	20 ± 1.0	1.85 ± 0.03	94 200 ± 6600
	tPA	1100 ± 160	0.36 ± 0.04	330 ± 58
Ac-FTAR-pNA	uPA	23 ± 1.0	1.52 ± 0.02	65 900 ± 3400
	tPA	480 ± 140	0.24 ± 0.03	500 ± 160
Bz-TAR-pNA	uPA	18 ± 1.0	1.63 ± 0.02	88 800 ± 4600
	tPA	1300 ± 570	0.46 ± 0.14	340 ± 180
Ac-QYAR-pNA	tPA	400 ± 87	0.78 ± 0.08	1900 ± 450
Ac-FYAR-pNA	tPA	590 ± 150	0.86 ± 0.12	1500 ± 420

the k_{cat}/K_M value for this substrate was almost 50-fold lower than the most preferred substrate for uPA (Table 3). Consistent with this, previous studies have reported that tPA cleaves a range of synthetic peptide substrates with low catalytic efficiency and K_M values in the millimolar range.[37] By contrast, the K_M value for cleavage of plasminogen by tPA is in the micromolar range and, in the presence of fibrin, the efficiency of tPA-mediated plasminogen activation is improved further due to a decrease in K_M by several orders of magnitude (k_{cat} is essentially unchanged).[5]

On comparing our findings for uPA and tPA, it is evident that each enzyme prefers a different set of tetrapeptide sequences, even though uPA and tPA share the same physiological cleavage site in plasminogen. To select the sequences for the peptide aldehyde library, we considered the specificity profile for each enzyme at the P1–P4 positions, as determined by positional scanning with chemically synthesised peptides.[27] Accordingly, the library was designed to include preferred sequences for both uPA and tPA. Screening the peptide library against each enzyme revealed that uPA and tPA both favour sequences with Ala or Gly at P2. However, at P3 and P4, uPA preferred sequences with P3 Thr and P4 Gly, whereas tPA preferred P3 Tyr and P4 Gln or Phe. Interestingly, the optimal sequences for uPA (GTAR) and tPA (QYAR) described in our study are similar to previously identified sequences for each enzyme, including in phage display screens. For uPA, the most preferred sequence identified by phage display was GSGR[29] and D-SAR has been widely used to design uPA inhibitors. For tPA, PFGR was the best sequence identified by phage display.[38] Although P4 Pro was not included in our peptide library, several peptides with the P3–P1 sequence FGR were screened, together with the uPA-favoured peptide GSGR, but these peptides were outperformed by new sequences that showed higher inhibitory activity for each enzyme.

In addition to preferring different tetrapeptide sequences, we found that the activity of the tetrapeptide substrates and inhibitors for uPA was noticeably different to tPA. The optimal sequences for uPA produced potent peptide aldehyde inhibitors with K_i values as low as 18 nM (Table 2), which is in a similar range to lead peptide aldehyde inhibitors from previous studies, including $BnSO_2$-D-SAR-H,[20] and other types of peptide-like inhibitors.[18,39] Additionally, these sequences yielded high efficiency peptide-pNA substrates that were cleaved with similar k_{cat}/K_M values to commercially available substrates ($1\times10^5\ M^{-1}s^{-1}$).[35,36] However, the inhibitors screened against tPA generally displayed weak activity ($IC_{50}>10\ \mu M$, Figure 2) and substrates based on the most preferred sequences were cleaved with k_{cat}/K_M values that were 50-fold lower compared to the optimal substrates for uPA. This finding is consistent with a previous study that examined the activity of tPA against peptide substrates with different amino acids at the P3 position based on X-Gly-Arg-pNA.[37] For substrates comprised of L-amino acids, the most preferred sequence was FGR-pNA (k_{cat}/K_M $1.4\times10^3\ M^{-1}s^{-1}$), which was cleaved with a similar k_{cat}/K_M value to Ac-QYAR-pNA (Table 3). Interestingly, improvements in k_{cat}/K_M that exceeded tenfold were identified when L-amino acids at P3 were replaced with their corresponding D-enantiomers.[37]

In conclusion, we have identified new preferred P4–P1 sequences for uPA and tPA using a sequence-defined peptide aldehyde inhibitor library. These tetrapeptide inhibitors showed high activity against uPA and the corresponding peptide-pNA substrates were cleaved with high catalytic efficiency, whereas tetrapeptide inhibitors and synthetic substrates showed weak activity against tPA. The synthetic substrates developed in our study have low K_M and high catalytic efficiency towards uPA and can potentially discriminate between uPA and tPA. These findings provide new insights for future studies on uPA and tPA inhibitor engineering and development.

Experimental Section

The serine protease domains of human uPA (residues 164–431) and human tPA (residue 297–562) were expressed in Expi293 cells as detailed in the Supporting Information.[40] Fmoc (9-fluorenylmethyl carbamate) N-protected amino acids were from Chem-Impex or CSbio unless stated. Reagents and solvents used in peptide synthesis and reverse-phase HPLC were provided by Merck unless stated. Peptide aldehydes were synthesised on H-Arg(Boc)2-H NovaSyn TG resin (Merck, 0.21 $mmol\,g^{-1}$), whereas peptide-pNA substrates were synthesised on 2-chlorotrityl chloride resin derivatised with 8 equiv of *para*-phenylenediamine (Sigma Aldrich) using standard Fmoc chemistry (full details are given in the Supporting Information). Protease inhibition assays were performed using the substrate Z-Pyr-Gly-Arg-MCA (Peptide Institute Inc.) and K_i values were determined by nonlinear regression using the Morrison equation and GraphPad Prism 6 software. Substrate kinetic assays were performed using peptide-pNAs and Michaelis–Menten kinetic constants were determined by nonlinear regression using GraphPad Prism 6. Full details for inhibition and substrate kinetic assays are provided in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: enzymes · inhibitors · peptides · plasminogen activation system · serine proteases

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