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SHORT COMMUNICATION

Tripeptides with C-Terminal Arginine as Potential Inhibitors of Urokinase

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Abstract We synthesized and tested ten peptides with the molecular structure being H–D-Ser–AA–Arg–OH for their effect on the amidolytic activities against urokinase, thrombin, trypsin, plasmin, tissue plasminogen activator and kallikrein. The inserted amino acid in each peptide was either leucine, norleucine, izoleucine, valine, norvaline, α-metyloalanine, α-aminobutanoic acid, homoleucine, tert-leucine or neoglycine. H–D-Ser–NVal–Arg–OH (compound **4**) was the most active inhibitor of urokinase plasminogen activator with a K_i value of 0.85 μM. Compound **4** showed cytotoxic effect against MDA-MB-231 and DLD cell lines, respectively, with IC_{50} values of 25 and 19 μM. Synthesised compounds did not have activity against MCF-7 cancer cells. These peptides were nontoxic against pig's erythrocytes in vitro.

Keywords Urokinase inhibitor · Anti-amidolytic activity · Low molecular weight peptides

Introduction

Urokinase plasminogen activator (uPA), a trypsin-like serine protease, plays an important role in several biological processes including tissue remodeling, cell migration and matrix degradation (Irigoyen et al. 1999; Syrovets and Simmet 2004). The primary role of uPA is converting plasminogen into its active form plasmin, a broad spectrum serine protease, which participates in the fibrinolytic cascade (Collen 2001; Rijken and Sakharov 2001). uPA and plasmin are strongly associated with tumor cells by influencing tumor initiation, proliferation, migration, invasion, metastasis and apoptosis (Andreasen et al. 1997; Duffy 2004; Mazar et al. 1999). Both these enzymes directly promote proteolysis. However, plasmin acts also indirectly through the activation of matrix metaloproteases. Overexpression of uPA has been found in various malignant tumors, especially in the gastrointestinal, respiratory, genitourinary systems, and in bones, bone marrow, skin, breast and brain (Dass et al. 2008). High levels of urokinase are correlated with enhanced tumor invasiveness, metastasis and poor prognosis in certain cancers (Sidenius and Blasi 2003).

The inhibition of plasminogen activation by uPA appears to be an attractive approach to cancer therapy, especially against tumor growth and metastasis (Rockway et al. 2002; Rockway 2003; Schweinitz et al. 2004). In 1997, Ke et al. analyzed a peptide library and selected the primary peptide sequence SGRSA (from position P3 to P2) as the substrate of uPA and tPA. Zeslawska et al. (2000, 2003) investigated the structure–function relationship of certain peptides such as phenethyl-sufonamidino-D-seryl-L-alanyl-L-argininal, and peptidomimetic inhibitors of uPA (e.g., benzamidine, amiloride, 2,4,6-triisopropyl-phenylsulfonyl-L-(3-amidino)-phenylalanine-piperazine-*N*-β-alanine). Studies have demonstrated that the S1 pocket of uPA is the critical site for interaction with inhibitors. The P1 positively charged residue of an inhibitor (arginine, benzamidine or phenylguanidine) makes a salt bridge with Asp189 in the S1 pocket of uPA. The P2 residue of the potential inhibitor, which should be hydrophobic, is accommodated in S2 side. When the P3 position is

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occupied by an unnatural D-amino acid, the side-chain projects into the S4 pocket. The residue of D-Ser could form a favorable interaction with His-99 in the S4 site of uPA.

Recently, we described a series of peptide analogs containing arginine as inhibitors of urokinase. The first series was H(Ac)–D-Ser–Ala–(Gly)–Arg–OH(NH_2) (Markowska et al. 2008) and the most active inhibitor of urokinase was H–D-Ser–Gly–Arg–OH with an IC_{50} value of 1 mM. The second series of peptide analogs of arginine we synthesized were the peptides of the general formula H–D-Ser–Ala–Arg–NH–X (Markowska et al. 2010), where X = $(CH_2)_n$–NH_2, n = 2–9, $(CH_2)_m$–OH, m = 2–4. H–D-Ser–Ala–Arg–NH–$(CH_2)_5$–NH_2 inhibited urokinase with a K_i value of 6.3 μM. The Lineweaver–Burke analysis of the H–D-Ser–Ala–Arg–NH–$(CH_2)_5$–NH_2 compound proved this compound to be a competitive inhibitor of urokinase. The third series of compounds with D-Ser–Ala–Arg sequence were *N*-sulfonylamides peptides (Markowska et al. 2010). 2,4,6-triisopropylphenyl–SO_2–D-Ser–Ala–Arg–OH was the most selective inhibitor of urokinase and α-tolyl–SO_2–D-Ser–Ala–Arg–OH was the most active inhibitor of uPA with a K_i value of 24 μM.

The purpose of this paper was the synthesis and the amidolytic activity of 10 novel peptides against urokinase, thrombin, trypsin, plasmin, tissue plasminogen activator (t-PA) and kallikrein in vitro. We also tested the hemolytic activity of the peptides against swine erythrocytes and the anti-tumor activity against the following human breast cancer cells: standard MCF-7, estrogen-independent MDA-MB-231 and colorectal adenocarcinoma tumor DLD cell line.

Materials and Methods

The inserted amino acid (AA) in each of the novel peptides was either leucine, norleucine, izoleucine, valine, norvaline, α-metyloalanine, α-aminobutanoic acid, homoleucine, tert-leucine or neoglycine. Table 1 represents the identity of the inserted amino acid in each of the 10 peptides synthesized from X–D-Ser–AA–Arg–OH.

Source of Reagents

Fmoc–Arg(Pbf)–OH (Fmoc = 9-fluorenylmethyloxycarbonyl, Pbf = pentamethyl-dihydrobenzofuran), chloranil, acetaldehyde, HOBt = 1-hydroxybenzotriazole were purchased from Fluka (Schnelldorf, Germany). Fmoc–D-Ser(t-Bu)–OH (t-Bu = t-butyl) and 2-chlorotrityl chloride resin were purchased from Merck (Novabiochem, Darmstadt, Germany). TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, piperidine, TBTU = tetrafluoroborate salt of the O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, DIC = diisopropylcarbodiimide, NMP = 1-methyl-2-pirrolidon, Fmoc–L-Leu–OH, Fmoc–L-NLe–OH, Fmoc–L-Ile–OH, Fmoc–L-Val–OH, Fmoc–L-NVa–OH, Fmoc–Aib–OH, Fmoc–L-2Abu–OH, Fmoc–L-HLeu–OH, Fmoc–L-TLe–OH, Fmoc–L-NptGly–OH were obtained from Iris Biotech GmbH (Marktrewitz, Germany). DCM = dichloromethane and DMF = dimethylformamide were the products of Chempur (Piekary Slaskie, Poland) DCM was used without further purification. DMF was distillated over ninhidrin and stored under molecular sieves 4A. HPLC solvent acetonitrile was purchased from Merck (Darmstadt, Germany). Urokinase, trypsin, kallikrein, amiloride and Bzl–L-Arg–pNA·HCl (Bzl = benzyl) were purchased from Sigma (Schnelldorf, Germany). Plasmin, S-2444 (pyro-Glu–Gly–Arg–pNA·HCl), S-2238 (H–D-Phe–Pip–Arg–pNA), S-2251 (H–D-Val–Leu–Lys–pNA), S-2266 (H–D-Val–Leu–Arg–pNA·2HCl and S-2288 (H–D-Ile–Pro–Arg–pNA) were obtained from Chromogenix (Milano, Italy). Thrombin and phosphate buffered saline (PBS) were purchased from Lubelska Wytwórnia Szczepionek (Lublin, Poland). t-PA was obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany).

Table 1 Amino acid insertion in novel peptides

Peptide	Inserted amino acid
1	α-Metyloalanine
2	α-Aminobutanoic acid
3	Valine
4	Norvaline
5	Leucine
6	Norleucine
7	Izoleucine
8	tert-Leucine
9	Homoleucine
10	Neoglycine

Peptide Synthesis

The peptides shown in Table 1 were synthesized manually using the standard Fmoc-based strategy (Chan and White 2000). Fmoc deprotection steps were carried out with 20% (v/v) piperidine in DMF/NMP (1:1) for 15 min. The coupling reactions of Fmoc amino acids were performed in DMF/NMP/DCM (1:1:1) using a molar ratio of amino acid/DIC/HOBt/resin 3:3:3:1. In the case of the coupling of Fmoc–D-Ser(t-Bu)–OH molar ratio of amino acid/TBTU/HOBt/DIPEA/resin was 2:2:2:4:1. The reactions were monitored with the Steward chloranil test. The cleavage from the resin was carried out with TFA/water (95/5). After 2.5 h stirring, the resin was filtered and washed with TFA.

The combined filtrates were concentrated under reduced pressure. The crude peptide was washed with cold diethyl ether, filtered, dissolved in glacial acetic acid and lyophilized.

The Shimadzu LC-10A system (Smith and Hanly 1997; Hartmann et al. 2009) (Shimadzu Europa GmbH, Duisburg, Germany) was used for analytical and semipreparatory HPLC (Phenomenex C18, Jupiter 90A, 4 µm, 250 × 10 mm; Phenomenex C18, Jupiter 300A, 5 µm, 250 × 4 mm; solvents: A, 0.1% aqueous TFA; B, 0.1% TFA in acetonitrile, gradient 0% B to 100% B in A in 30 min, flow rate 1 ml/min, monitored at 220 nm). The major peak fraction was pooled and lyophilized. The molecular weight determination was performed by mass spectrometry using a Bruker Daltonics Esquire 6000 (Bruker Daltonik GmbH, Leipzig, Germany) with electrospray ionization (ESI), (Table 2).

Enzymatic Investigations

Determination of amidolytic activity was performed as previously described (Okada et al. 1988). A detailed description of the method is given below. Buffer and 0.1 cm^3 of enzyme solution was added to 0.2 cm^3 of examined compound (**1–10**) (as control 0.15 M NaCl). The buffer and the enzyme solution included the following constituents:

a. Tris buffer: 0.6 cm^3 (pH 8.8), enzyme: urokinase (50 units/cm^3), synthetic substrate: S-2444 (0.1 cm^3, 3 mM/dm^3);
b. Tris buffer: 0.5 cm^3 (pH 8.4), enzyme: thrombin (1 units/cm^3), synthetic substrate: S-2238 (0.2 cm^3, 0.75 mM/dm^3);
c. Tris buffer: 0.5 cm^3 (pH 7.4), enzyme: plasmin (0.4 units/cm^3), synthetic substrate: S-2251 (0.2 cm^3, 3 mM/dm^3);
d. borane buffer: 0.5 cm^3 (pH 7.5), enzyme: trypsin (0.4 units/cm^3), synthetic substrate: Bzl–L-Arg–pNA·HCl (0.2 cm^3, 8 mM/dm^3);
e. Tris buffer: 0.6 cm^3 (pH 9.0), enzyme: kallikrein (3 units/cm^3), synthetic substrate: S-2266 (0.1 cm^3, 7.5 mM/dm^3);
f. Tris buffer: 0.6 cm^3 (pH 8.4), enzyme: t-PA (1.67 mg/cm^3), synthetic substrate: S-2288 (0.1 cm3, 10 mM/dm^3).

The mixture was incubated for 3 min at 37°C, followed by the addition of synthetic substrate solution in the same buffer. After 20 min of incubation, the reaction was stopped by adding 0.1 cm^3 of 50% acetic acid and the absorbance of the released p-nitroaniline was measured at 405 nm. As shown in Table 3, each value represents the average of a triplicate determination. The IC_{50} value for each peptide was considered as the concentration of the inhibitor, which decreased the absorbance at 405 nm by 50%, compared with the absorbance measured under the same conditions without the inhibitor. Also, K_i was calculated for each peptide from IC_{50} based on Cheng–Prusoff equation (Cheng and Prusoff 1973). The results are shown in Table 3.

Our results were compared with the data obtained by Tamura et al. (2000) for 2-phenethyl–SO_2–D-Ser–Ala–Arg–al, the irreversible urokinase inhibitor with the same tripeptide sequence. The determination methods were identical.

Tissue Culture

All studies were performed on MCF-7, MDA-MB-231 and DLD cells lines were purchased from American Type Culture Collection, (Rockville, MD). The cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS), 2 mmol/ml glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Table 2 Analytical data of the synthesized peptides

Peptide no.	Yield (%)	Retention time (min)	Melting point (°C)	MW	$[M^+H]^+$
1	46	8.93	223–225	346.4	347.7
2	47	8.89	231–234	346.4	347.6
3	45	9.72	211–212	360.4	361.7
4	45	9.99	243–245	360.4	361.3
5	47	11.32	219–220	374.4	375.6
6	56	11.27	232–233	374.4	375.5
7	43	10.99	228–229	374.4	375.6
8	59	10.93	197–199	374.4	375.6
9	45	13.08	221–225	388.5	389.4
10	58	12.10	138–139	388.4	389.5

Table 3 Inhibition of amidolytic activity of enzymes against synthetic peptides with the general H–D-Ser–AA–Arg–OH formula

No.	AA	K_i (μM)				
		Urokinase S-2444	Thrombin S-2238	Trypsin Bzl–L-Arg–pNA·HCl	Plasmin S-2251	t-PA S-2288
1.	α-Metyloalanine	n.i.	n.i.	n.i.	59.09 ± 4.73	n.i.
2.	α-Aminobutanoic acid	n.i.	1.19 ± 0.09	4.73 ± 0.38	2.54 ± 0.20	n.i.
3.	Valine	182.92 ± 14.63	n.i.	4.48 ± 0.36	163.63 ± 13.09	n.i.
4.	Norvaline	0.85 ± 0.07	n.i.	n.i.	4.36 ± 0.35	n.i.
5.	Leucine	219.51 ± 17.56	1.1 ± 0.09	4.73 ± 0.38	27.27 ± 2.18	154.54 ± 12.36
6.	Norleucine	4.45 ± 0.34	n.i.	n.i.	40.9 ± 3.27	n.i.
7.	Izoleucine	158.53 ± 12.68	n.i.	0.77 ± 0.06	1.81 ± 0.15	19.09 ± 1.53
8.	tert-Leucine	26.62 ± 2.15	1.43 ± 0.11	n.i.	31.81 ± 2.55	n.i.
9.	Homoleucine	n.i.	n.i.	3.74 ± 0.30	n.i.	n.i.
10.	Neoglycine	n.i.	n.i.	n.i.	n.i.	163.63 ± 13.09
	2-Ph–$(CH_2)_2$–SO_2–D-Ser–Ala–Arg–al	3 nM (Tamura et al. 2000)	–	–	–	–
	Benzyl–SO_2–D-Ser–4-amidinobenzylamid	7.7 nM (Zeslawska et al. 2003)	–	–	–	–
	Amiloride	7 μM (Zeslawska et al. 2003)				

n.i. No inhibition was observed at maximum concentration (0.02 M)

Cytotoxicity Assay

The toxicity of the evaluated compounds was determined by the method of Plumb et al. (Plumb et al. 1989). MCF-7, MDA-MB-231 and DLD cells were maintained as described above. After 48 h of incubation of the cells with synthesized peptides, the medium was discarded and the cells were rinsed three times with PBS. The cells were then incubated for 4 h in 2 ml of PBS with 50 ml of MTT (5 mg/ml). After removal of the medium, the cells were lysed in 200 ml of DMSO with 20 ml of Sorensen's buffer (0.1 M glycine with 0.1 M NaCl, pH 10.5). The absorbance was measured at 570 nm. The cytotoxic activity towards MDA-MB-231 and DLD of synthesized peptides was calculated as percentage of nonviable cells and the IC_{50} value was estimated from logarithm curves as shown in Table 4.

Table 4 The nonviability of DLD and MDA-MB-231 cells treated for 24 h with different concentrations of the synthesized inhibitors

Compound no.	IC_{50} (μM) (% of control ± 2)	
	DLD	MDA-MB-231
1	32	54
2	43	76
3	55	48
4	19	25
5	56	119
6	52	84
7	59	80
8	57	81
9	n.e.	n.e.
10	n.e.	n.e.
Amiloride	62.1	126

n.e. No cytotoxic effect was observed in maximum concentration (250 μM)

[a] Mean values ± SD from three independent experiments done in duplicate

Hemolytic Activity

Pig's fresh red blood cells (p-RBC) were washed three times with PBS (35 M phosphate buffer/0.15 mM NaCl, pH 7.4) and were centrifuged at 1000×*g* for 10 min to remove plasma and the buffy coat. Erythrocytes in suspension were incubated with various concentrations of peptides for 1 h at 37°C. The final erythrocyte concentration was 5% v/v. After the centrifugation (1000×*g* for 10 min), 100 μl of the supernatant was transferred into sterilized 96-well plates, where hemoglobin release was monitored, using Infinite M200 plate reader and measuring the absorbance at 414 nm. Zero hemolysis (blank), hemolysis with amiloride as reference compound for synthesised peptides and 100% hemolysis, which consisted of p-RBC suspended in PBS and 0.1% Triton-X-100, were determined respectively. The percentage of hemolysis was calculated with the following formula: %hemolysis = (Abs_{414nm} in the peptide solution in PBS/Abs_{414nm} in 0.1% Triton-X-100 in PBS) × 100.

Results and Discussion

We obtained ten new peptides as potential inhibitors of urokinase by the manual solid phase synthesis and tested them for the effect on the amidolytic activities of urokinase, thrombin, trypsin, plasmin, t-PA and kallikrein. These peptides did not influence the enzymatic activity of kallikrein.

The results indicated that the concentration up to 1000 μg/ml of the synthesized peptides did not lyse erythrocytes.

The synthesized peptides were not selective inhibitors of urokinase. Peptides **1** (H–D-Ser–Aib–Arg–OH) and **2** (H–D-Ser–Abu–Arg–OH), containing two carbon atoms in their amino acid side chains inhibited the activity of plasmin. Peptides with three and four carbon atoms in the amino acid side chains (H–D-Ser–Val–Arg–OH **3**, H–D-Ser–NVal–Arg–OH **4**, H–D-Ser–Leu–Arg–OH **5**, H–D-Ser–NLeu–Arg–OH **6**, H–D-Ser–ILeu–Arg–OH **7**, H–D-Ser–tLeu–Arg–OH **8**) inhibited plasmin and urokinase. Tripeptides **9** and **10**, containing homoleucine and neoglycine respectively as P2 residue, were non active inhibitors of plasmin and urokinase. The most active peptide against urokinase was H–D-Ser–NVal–Arg–OH **4** with a K_i value 0.85 μM.

The obtained values of K_i were higher than K_i of the standard inhibitors. However, 2-phenethyl–SO_2–D-Ser–Ala–Arg–H was an alkylating agent and irreversibly inhibited urokinase by forming a covalent adduct with an active site of the enzyme (Zeslawska et al. 2003; Tamura et al. 2000). Benzyl–SO_2–D-Ser–4-amidinobenzylamid with $K_i = 0.0077$ μM inhibitory activity was a non-peptidic inhibitor of urokinase. 4-Amidinobenzylamine was a decarboxylated arginine mimetic, which has been widely used for the development of urokinase inhibitors (Rockway 2003; Schweinitz et al. 2004). The previous analysis of the effect of H–D-Ser–Ala–Arg–NH–$(CH_2)_5$–NH_2 on the activity of urokinase showed that the derivatives with these kinds of sequence reversibly and competitively inhibited urokinase.

The peptides **1**–**10** were tested in vitro for their antitumour activity in human breast cancer cells: standard MCF-7, estrogen-independent MDA-MB-231 and colorectal adenocarcinoma tumor DLD line. Peptides **9** and **10** were not active in vivo. The most interesting peptide was compound **4** with an IC_{50} values of 19 μM for DLD cells and 25 μM for MDA-MB-231 cells, which was the most selective inhibitor of urokinase. All potential inhibitors were insignificantly less cytotoxic to MDA-MB-231 and DLD cells than amiloride. Amiloride, which is a potassium-sparing diuretic drug, has been shown to inhibit uPA with $K_i = 7$ μM and to have antitumor activity in vivo (Zeslawska et al. 2003).

The synthesized peptides did not influence MCF-7 cancer cells. It was found that the proteolytic activity of uPA is closely related to cell-surface events when incubated with the breast cancer cells. MCF-7 had low uPAR/uPA-expressing and low plasminogen-binding, whereas MDA-MB-231 had high uPAR/uPA expressing and high plasminogen-binding (Stillfried et al. 2007). Thus, the influence of the synthetic peptides on the cytotoxic effect of MCF-7 cells could be insignificant.

We hope that our studies provided information about the structure–activity and may be used for synthesizing more active enzyme inhibitors and potential antitumor agents.

Conclusion

Our investigation of the structure–activity relationship of a series of new peptide sequences as potential urokinase inhibitors reveals that the obtained H–D-Ser–NVal–Arg–OH was the most active inhibitor of uPA with K_i 0.85 μM value. The compound showed cytotoxic effects against MDA-MB-231 cell lines with a IC_{50} 25 μM value and with a IC_{50} 19 μM value against DLD cell lines. The examined compound did not influence MCF-7 cancer cells.

References

Andreasen PA, Kjřller L, Christensen L, Duffy MJ (1997) The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72:1–22

Chan WC, White PD (2000) Fmoc solid phase peptide synthesis. Oxford University Press, New York

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor, which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Collen D (2001) Ham-Wasserman lecture: role of the plasminogen system in fibrin-homeostasis and tissue remodeling. Hematology 1:1–9

Dass K, Ahmad A, Azmi AS, Sarkar SH, Sarkar FH (2008) Evolving role of uPA/uPAR system in human cancers. Cancer Treat Res 34:122–136

Duffy MJ (2004) The urokinase plasminogen system: role in malignancy. Curr Pharm Des 10:39–49

Hartmann BM, Kaar W, Yoo IK, Lua LHL, Falconer RJ, Middelberg APJ (2009) The chromatography-free release, isolation and purification of recombinant peptide for fibril self-assembly. Biotechnol Bioeng 104:973–985

Irigoyen JP, Muňoz-Cánoves P, Montero L, Koziczak M, Nagamine Y (1999) The plasminogen activator system: biology and regulation. Cell Mol Life Sci 56:104–132

Ke SH, Coombs GS, Tachias K, Corey DR, Madison EL (1997) Optimal subsite occupancy and design of a selective inhibitor of urokinase. J Biol Chem 272:20456–20462

Markowska A, Bruzgo I, Midura-Nowaczek K (2008) Effects of tripeptides on the amidolytic activities of urokinase, thrombin, plasmin and trypsin. Int J Pept Res Ther 14:215–218

Markowska A, Bruzgo I, Midura-Nowaczek K (2010a) Synthesis and activity of amides of tripeptides as potential urokinase inhibitors. J Enz Inh Med Chem 25:139–142

Markowska A, Bruzgo I, Midura-Nowaczek K (2010b) Synthesis and activity of N-sulfonylamides of tripeptides as potential urokinase inhibitors. Prot Pept Lett 17:1300–1304

Mazar AP, Henkin J, Goldfarb RH (1999) The urokinase plasminogen activator system in cancer: implications for tumor angiogenesis and metastasis. Angiogen 3:15–23

Okada Y, Tsuda Y, Teno N, Wanaka K, Bohgaki M, Hijikata-Okunomiya A, Naito T, Okamoto S (1988) Synthesis of active center-directed peptide inhibitors of plasmin. Chem Pharm Bull 36:1289–1297

Plumb JA, Milroy R, Kaye SB (1989) Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res 49:4435–4440

Rijken DC, Sakharov DV (2001) Basic principles in thrombolysis: regulatory role of plasminogen. Thromb Res 103:S41–S49

Rockway TW (2003) Small molecules inhibitors of urokinase-type plasminogen activator. Expert Opin Ther Patents 13(6):773–786

Rockway TW, Nienaber V, Giranda VL (2002) Inhibitors of protease domain of urokinase-type plasminogen activator. Curr Pharm Design 8:2541–2558

Schweinitz A, Steimetzer T, Banke IJ, Arlt MJE, Stürzebecher A, Schuster O, Geissler A, Giersiefen H, Zesławska E, Jacob U, Krűger A, Stűrzebecher J (2004) Design of novel and selective inhibitors of urokinase-type plasminogen activator with improved pharmacokinetic properties for use as antimetastatic agents. J Biol Chem 279:33613–33622

Sidenius N, Blasi F (2003) The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. Cancer Metastasis Rev 22:205–222

Smith DD, Hanly AM (1997) Purification of synthetic peptides by high performance liquid chromatography. Neuropept Protoc 73:75–87

Stillfried GE, Saunders DN, Ranson M (2007) Plasminogen binding and activation at the breast cancer cell surface: the integral role of urokinase activity. Breast Cancer Res 9:R14

Syrovets T, Simmet T (2004) Novel aspects and new roles for the serine protease plasmin. Cel Mol Life Sci 61:873–885

Tamura S, Weinhouse MI, Roberts CA, Goldman EA, Masukawa K, Anderson SM, Cohen CR, Bradbury AE, Bernardino VT, Dixon SA, Ma MG, Nolan TG, Brunck TK (2000) Synthesis and biological activity of peptidyl aldehyde urokinase inhibitors. Biorg Med Chem Lett 10:983–987

Zeslawska E, Schweinitz A, Karcher A, Sondermann P, Sperl S, Stürzebecher J, Jacob U (2000) Crystals of the urokinase type plasminogen activator variant beta(c)-uPA in complex with small molecule inhibitors open the way towards structure-based drug design. J Mol Biol 301:465–475

Zeslawska E, Jacob U, Schweinitz A, Coombs G, Bode W, Madison EJ (2003) Crystals of urokinase type plasminogen activator complexes reveal the binding mode of peptidomimetic inhibitors. J Mol Biol 328:109–118