Synthesis and Activity of N-Sulfonylamides of Tripeptides as Potential Urokinase Inhibitors

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Abstract: Twelve peptides of the general X-SO_2-D-Ser-Ala-Arg-OH formula (where X = methyl, phenyl, α-tolyl, p-tolyl, 4-methylbenzyl, 1-naphtyl, 2-naphtyl, 4-chlorophenyl, 4-bromophenyl, 2-mesityl, 2,4,6-triisopropylphenyl, 4-acetamidophenyl) were obtained and tested for their effect on the amidolytic activities of urokinase, thrombin, trypsin, plasmin, t-PA and kallikrein. 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 K_i value 24 μM. The compounds were tested for their *in vitro* antitumour activity in the following human breast cancer cells: standard MCF-7 and estrogen-independent MDA-MB-231. Four of the synthesized peptides showed cytotoxic effects against MDA-MB-231 cell lines in the range from 2.9 to 8.5 μM. The examined compound did not influence to MCF-7 cancer cells. The synthesized peptides were nontoxic to pig's erythrocytes.

Keywords: Urokinase inhibitor, low molecular peptide.

INTRODUCTION

The urokinase plasminogen activator system consists of the serine protease urokinase (uPA), its cell surface-associated receptor (uPAR), plasminogen activator inhibitors (PAIs) and the proenzyme plasminogen (Plg). uPA is responsible for the Plg activation to plasmin (Plm) by the Arg561-Val562 bond hydrolysis in Plg.

Plm, the key enzyme of fibrinolysis, is a non-specific trypsin-like protease, which cleaves after numerous Lys or Arg bonds. It attacks fibronectin, fibrin/fibrinogen, clotting factors V/Va and VIII/VIIIa, latent TGF-β, IGF binding proteins and the zymogen forms of several metalloproteases [1, 2]. In contrast, uPA is a highly specific serine protease, which catalyses by cleaving single Arg-X or Lys-X bonds for example the hepatocyte growth factor, fibronectin, diphtheria toxin, uPAR and uPA itself [3]. The two-chain active form of uPA is activated from a single chain precursor (prouPA) by plasmin or possibly via enzymes commonly enriched in cancer cells such as thiol cathepsins. uPA is unique in having its own high affinity cell-surface receptor uPAR. The urokinase receptor is focalized in the cell-cell connection and at the edge of invading cells. Thus, the uPA system plays a pivotal role in degradating and regenerating the basement membrane which leads directly to tissue remodelling, invasiveness and angiogenesis [4-6]. The binding of uPA to uPAR also initiates signalling cascades that does not require the uPA catalytic activity but only receptor occupancy. The expression of uPA and uPAR has been demonstrated in essentially every cancer type, such as gastric, colorectal, ovarian, breast, endometrial and prostate cancer [7].

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There are currently no inhibitors of the uPA system in human cancer trials. However, several small-molecule inhibitors of uPA have been described in the literature [8]. Kawada and Umezawa described the Pyr-Leu-Arg-H leupeptin analogue which showes activity against both uPA and plasmin and inhibits the invasion of HT1080 tumor cells *in vitro* [9]. The H-D-Ser-Ala-Arg-H inhibitor reported by Tamura *et al.* contains unusual but useful D-serine as P3 residue which is normally oriented into solvent, does not interact with trypsin-like proteinases and is not hydrolized *in vivo* [10]. However, aldehyde derivatives are alkylating agents and they irreversibly inhibit uPA by forming a covalent adduct with histidine in the active site of the enzyme.

On the basis of this peptide sequence we described a series of tripeptides as potential inhibitors of urokinase [11]. The compounds with a free amino group: H-D-Ser-Ala-Arg-OH and H-D-Ser-Gly-Arg-OH inhibit urokinase, thrombin, plasmin and trypsin. The N-acetylated tripeptides show some selectivity: Ac-D-Ser-Ala-Arg-OH inhibits thrombin and trypsin but Ac-D-Ser-Gly-Arg-OH inhibits urokinase and plasmin. The N-acetylated tripeptides were in general less active than the corresponding compounds with a free amino group. H-D-Ser-Ala-Arg-CONH-$(CH_2)_5$-NH_2 containing 5-aminopentylamide inhibits plasmin and urokinase. Lineweaver-Burke analysis for the H-D-Ser-Ala-Arg-CONH-$(CH_2)_5$-NH_2 compound proved that it competitively inhibits urokinase [12].

We presented the synthesis of peptides of the general X-SO_2-D-Ser-Ala-Arg-OH formula, where X = methyl, phenyl, α-tolyl, p-tolyl, 4-methylbenzyl, 1-naphtyl, 2-naphtyl, 4-chlorophenyl, 4-bromophenyl, 2-mesityl, 2,4,6-triisopropylphenyl, 4-acetamidophenyl. Six trypsin-like serine proteases were used to determine the amidolytic activity of potential inhibitors. Trypsin was used as a standard enzyme for this protease class, whereas thrombin, plasmin, t-PA (tissue plasminogen activator) and kallikrein were selected to predict a

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possible influence on blood coagulation and fibrynolysis. Preeliminary evaluation of the biological properties of the cytotoxic activity in MCF-7 and MDA-MB-231 cell lines of the synthesized tripeptides was determined. The toxicity to erythrocytes was investigated using pig's red blood cells.

MATERIALS AND METHODS

Materials

Fmoc-Arg(Pbf)-OH (Fmoc=9-fluorenylmethyloxycarbonyl, Pbf=penta-methyldihydrobenzofuran), Fmoc-Ala-OH, chloranil, acetaldehyde, HOBt=1-hydroxybenzotriazole, 2-chloritrityl chloride resin, methanesulfonyl chloride, benzenesulfonyl chloride, α-toluenesulfonyl chloride, p-toluenesulfonyl chloride, 4-methylbenzylsulfonyl chloride, 1-naphtalenesulfonyl chloride, 2-naphtalenesulfonyl chloride 4-chlorobenzenesulfonyl chloride, 4-bromobenzenesulfonyl chloride, 2-mesitylenesulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride, N-acetylsulfanilyl chloride were purchased from Fluka. Fmoc-D-Ser(t-Bu)-OH (t-Bu=-butyl) were purchased from Merck (Novabiochem). TFA=trifluoroacetic acid, DIPEA=diisopropylethylamine, piperidine, TBTU=O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate, DIC=diisopropylcarbodiimide, NMP=1-methyl-2-pirrolidon were obtained from Iris Biotech GmbH. DCM=dichloromethane and DMF=dimethylformamide were the products of Chempur. DCM was used without further purification. DMF was distillated over ninhidrin and stored under 4A molecular sieves. HPLC solvent - acetonitrile was purchased from Merck.

Urokinase, trypsin, kallikrein, amiloride and Bzl-L-Arg-pNA·HCl (Bzl=benzyl) were purchased from Sigma. Plasmin, S-2444 (pGlu-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. Thrombin and phosphate buffered saline (PBS) were purchased from Lubelska Wytwórnia Szczepionek. t-PA was obtained from Boehringer Inglheim.

Synthesis of Inhibitors

The peptides shown in Table **1** were synthesised manually using the standard Fmoc-based strategy. The 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 the molar ratio of amino acid/DIC/HOBt/resin (3:3:3:1) for the coupling of Fmoc-Arg(Pbf)-OH and Fmoc-Ala-OH. In the case of the coupling of Fmoc-D-Ser(t-Bu)-OH, the molar ratio of amino acid/TBTU/HOBt/DIPEA/resin was 2:2:2:4:1. The sulfonyl chlorides were used as 10 molar excess to resin with 20 molar excess of DIPEA to resin. The reactions were monitored with the Stewart chloranil test.

The cleavage from the resin was carried out with TFA/water (95:5). After stirring for 2.5 h, 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.

Table 1. Structure of Obtained Peptides: X-SO_2-D-Ser-Ala-Arg-OH

Compound	X
1	methyl
2	phenyl
3	α-tolyl
4	p-tolyl
5	4-methylbenzyl
6	1-naphtyl
7	2-naphtyl
8	4-chlorophenyl
9	4-bromophenyl
10	2-mesityl
11	2,4,6-triisopropylphenyl
12	4-acetamidophenyl

The Shimadzu LC-10A system was used for analytical and semipreparatic HPLC (Phenomenex C18, Jupiter 90A, 4 micron, 250 x 10mm; Phenomenex C18, Jupiter 300A, 5 micron, 250 x 4mm; 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 with electrospray ionization (ESI), (Table **2**).

Enzymatic Investigations

The determination of the amidolytic activity was perfomed as previously described [13]. The 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 the examined compound (**1-12**) (as control 0.15 M NaCl). The buffer and the enzyme solution included:

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 cm3 (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 cm3 (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 cm3 (pH 7.5),
Enzyme: trypsin (0.4 units/cm^3),

Table 2. Analytical Data of The Synthesized Compounds

No	Yield [%]	Retention Time [min.]	Melting Point [°C]	MW	$[M+H]^+$
1	45	19.3	80-81	410.4	411.5
2	46	12.9	82-83	472.5	473.4
3	56	13.8	60-61	486.5	487.5
4	43	14.2	127-128	486.5	487.5
5	57	15.2	65-66	500.6	501.7
6	61	15.9	92-93	522.6	523.7
7	31	18.9	149-150	522.6	523.8
8	59	14.7	95-96	506.9	507.8
9	29	15.4	86-87	551.4	552.6
10	58	16.4	138-139	514.6	515.4
11	56	21.7	162-163	598.8	599.7
12	48	20.8	95-97	529.6	530.9

Synthetic substrate: Bzl-L-Arg-pNA·HCl (0.2 cm^3, 8 mM/dm^3);

e) Tris buffer – 0.6 cm3 (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 cm3 (pH 8.4),

Enzyme: t-PA (1.67 mg/cm^3),

Synthetic substrate: S-2288 (0.1 cm^3, 10 mM/dm^3).

The mixture was incubated for 3 min at 37°C, then the synthetic substrate solution was added to 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. Every value represents the average of the triplicate determination. IC_{50} value was considered 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. K_i was calculated from IC_{50} based on the Cheng-Prusoff equation [14]. The results are given in Table **3**.

Tissue Culture

All studies were performed using MCF-7 and MDA-MB-231, breast cancer cell 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.

Cytotoxicity Assay

The toxicity of the evaluated compounds was determined by the method described by Plumb *et al.* [15]. MCF-7 and MDA-MB-231 cells were maintained as described above. After 48 h of incubation of the cells with drugs, the medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). Then the cells were incubated for 4 h in 2 ml of PBS with 50 ml of MTT (5 mg/ml). The medium was removed from the cells, and 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.

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 centrifugated at 1000 g for 10 min. to remove plasma and the buffy coat. Various concentrations of peptides were incubated with the erythrocyte suspension 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 with the use of the Infinite M200 plate reader by 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) x 100.

RESULTS AND DISCUSSION

Using the manual solid phase synthesis, we obtained twelve new compounds as potential inhibitors of urokinase. The examined compounds did not influence the enzymatic activity of kallikrein.

Table 3. Inhibition of X-SO_2-D-Ser-Ala-Arg-OH on The Amidolytic Activity of Enzymes

No	X	K_i [μM]				
		Urokinase S-2444	Thrombin S-2238	Trypsin Bzl-L-Arg-pNA·HCl	Plasmin S-2251	t-PA S-2288
1	methyl	n.i.	n.i.	3.99 ± 0.35	n.i.	n.i.
2	phenyl	n.i.	11.98 ± 0.96	n.i.	n.i.	n.i.
3	α-tolyl	24.4 ± 1.9	n.i.	44.9 ± 3.99	n.i.	n.i.
4	p-tolyl	146.3 ± 9.7	n.i.	14.9 ± 1.3	n.i.	n.i.
5	4-methylbenzyl	n.i.	14.4 ± 1.2	n.i.	109.1 ± 9.1	181.8 ± 14.5
6	1-naphtyl	n.i.	n.i.	19.95 ± 1.6	1272.7 ± 10	27.3 ± 2.2
7	2-naphtyl	121.9 ± 10.2	n.i.	n.i.	545.5 ± 36.4	181.8 ± 14.5
8	4-chlorophenyl	n.i.	n.i.	n.i.	181.8 ± 14	45.4 ± 3.8
9	4-bromophenyl	n.i.	7.2 ± 0.48	n.i.	272.7 ± 21.8	27.3 ± 2.2
10	2-mesityl	n.i.	n.i.	n.i.	n.i.	181.8 ± 14.5
11	2,4,6-triisopropylphenyl	390.2 ± 31.7	n.i.	n.i.	n.i.	n.i.
12	4-acetamidophenyl	n.i.	n.i.	n.i.	n.i.	n.i.
	2-Ph-$(CH_2)_2$--SO_2-D-Ser-Ala-Arg-al	3 nM [15]	-	-	-	
	Benzyl-SO_2-D-Ser-4-amidinobenzylamid	7.7 nM [18]				

Table 4. The Viability of MDA-MB-231 Cells Treated for 24 h With Different Concentrations of The Synthesised Inhibitors

Concentration [μM]	Nonviable Cells (% of Control ± 2)[a]				
	3	4	7	11	Amiloride
1	20	36	14	38	21
10	68	71	64	53	21
50	73	73	80	68	24
250	87	86	83	84	55
500	100	100	100	100	100
IC_{50}	6.9	2.9	8.5	5.2	62.1

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

Among the obtained compounds, 2,4,6-triisopropylphenyl-SO_2-D-Ser-Ala-Arg-OH (**11**) was the most selective one towards urokinase, and α-tolyl-SO_2-D-Ser-Ala-Arg-OH (**3**) was the most active inhibitor of uPA. Methyl-SO_2-D-Ser-Ala-Arg-OH was the selective inhibitor of trypsin and phenyl-SO_2-D-Ser-Ala-Arg-OH of thrombin.

The obtained values of K_i are higher than K_i of the earlier described inhibitors. However, 2-phenethyl-SO_2-D-Ser-Ala-Arg-H is an alkylating agent and irreversibly inhibits urokinase by forming a covalent adduct with an active site of the enzyme [10, 16]. Benzyl-SO_2-D-Ser-4-amidinobenzyl-amid with K_i = 0.0077 μM inhibitory activity, which is a close analog of **3**, is a non-peptidic inhibitor of urokinase. 4-Amidinobenzylamine is a decarboxylated arginine mimetic, and has been widely used for the devolopment of urokinase inhibitors [17, 18]. 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 derivatives with these kinds of sequence competitively inhibit urokinase [12].

The compounds **3**, **4**, **7** and **11** were tested for their *in vitro* antitumour activity in human breast cancer cells, standard MCF-7 and estrogen-independent MDA-MB-231. Their cytotoxic activity towards MDA-MB-231 is shown in Table **1** as the percentage of nonviable cells. All tested compounds

showed concentration-dependent activity. The most interesting compounds seem to be **4** with IC_{50} value 2.9 μM for MDA-MB-231 cells and compound **11**, which was the most selective inhibitor of urokinase and showed a cytotoxic effect in concentration 5.2 μM for MDA-MB-231 cells. All potential inhibitors are slightly more cytotoxic to MDA-MB-231 cells than the examined amiloride. Originally, amiloride was an antidiuretic agent, and it was later found that it inhibits uPA with K_i = 7μM and has an antitumor activity *in vivo* [16]. Further development of amiloride was limited by the lack of selectivity and weak solubility.

The examined compound did not influence MCF-7 cancer cells. It was found that the proteolytic activity of uPA is closely connected with cell-surface events at the breast cancer cell. MCF-7 cells have low uPAR/uPA-expressing and low plasminogen-binding, whereas MDA-MB-231 cells have high uPAR/uPA expressing and high plasminogen-binding [19]. Thus, the influence of the synthesized compounds on the cytotoxic effect of MCF-7 cells could be insignificant.

The toxicity to erythrocytes was investigated using pig's red blood cells. The results show that the concentration up to 1000 μg/ml of the synthesized peptides did not lyse erythrocytes.

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