Synthesis and Biological Activity of N-Sulfonyletripeptides with C-Terminal Arginine as Potential Serine Proteases Inhibitors

Agnieszka Markowska • Magdalena Bruzgo • Ewa Gorodkiewicz • Arkadiusz Surażyński

Abstract Tripeptides of the general X-SO₂-d-Ser-AA-Arg-CO-Y formula, where X = α-tolyl, p-tolyl, 2,4,6-triisopropylphenyl; AA = alanine, glycine, norvaline and Y = OH, NH-(CH₂)₅NH₂ were obtained and tested for their effect on the amidolytic activities of urokinase, thrombin, trypsin, plasmin, t-PA and kallikrein. The most active compound towards urokinase was PhCH₂SO₂-d-Ser-Gly-Arg-OH with Kᵢ value 5.4 µM and the most active compound toward thrombin was PhCH₂SO₂-d-Ser-NVa-Arg-OH with Kᵢ value 0.82 µM. The peptides were nontoxic against porcine erythrocytes in vitro. PhCH₂SO₂-d-Ser-Gly-Arg-OH showed cytotoxic effect against DLD cell lines with IC₅₀ values of 5 µM. For the highly selective determination of the interaction of some of the synthesised acids of tripeptides with urokinase and plasmin the Surface Plasmon Resonance Imaging sensor has been applied. These compounds bind to urokinase and plasmin in 0.05 mM concentration.

Keywords Urokinase inhibitor • Serine proteases inhibitor • Amidolytic activity • Cancer cells activity • Surface Plasmon Resonance Imaging

Introduction

The proteolytic degradation of the extracellular matrix is essential for the processes of tissue remodelling. These processes take place in a number of distinct physiological events in the healthy organism such as matrix degradation, cell motility, angiogenesis and wound healing, as well as in the critical mechanisms in tumor invasion and metastasis. The plasminogen activation system has an important role as a proteolytic cascade in these degradation reactions (Irigoyen et al. 1999). The enzymatic system consists of urokinase receptor (uPAR), urokinase plasminogen activator (uPA) and plasminogen activator inhibitors: type-1 (PAI-1) and type-2 (PAI-2). uPAR is a cell membrane-anchored binding protein. uPA accumulates plasminogen activation activity at cell surfaces (Ossowski and Aguirre-Ghiso 2000). uPA is the primary cellular activator which converts the pro-enzyme plasminogen into its active form plasmin (Irigoyen et al. 1999). Plasmin is a broad spectrum serine protease which dissolve fibrin blood clots, activates metalloproteinases, degrades basement membrane and many blood plasma proteins (Longstaff and Thelwell 2005).

Numerous studies have shown the relationship between the level of expression of the uPA enzyme system and the poor prognosis in brain, colon, stomach, ovary, breast and kidney cancers (Duffy 2004; Sidenius and Blasi 2003; Dass et al. 2008). Urokinase has become an attractive therapeutic target in a variety of tumor.
Urokinase inhibitors are divided into 3 classes: inhibitors of the uPA–uPAR interaction, inhibitors of the uPA–PAI-1 interaction and inhibitors of uPA proteolytic activity.

uPA–uPAR interaction cyclic or linear inhibitors are based on a specific amino acid sequence which comes from the growth factor domain (GFD) of urokinase. These peptides contains from 10 to 32 amino acids from this region of uPA (Ploug et al. 2001; Goodson et al. 1994).

Corvas has described several substituted phenylpropionic acid derivatives as effective PAI-1 antagonists (Tamura et al. 2000). High levels of PAI-1 have been suggested as one of the factors that contribute a poor prognosis for cancer patients (Binder and Mihaly 2008).

Many small molecules have been reported to inhibit the proteolytic activity of uPA. These peptide-based compounds include the central aromatic or heteroaromatic (thiophen, indole, pyridine) core (Klinghofer et al. 2001; Schweinitz et al. 2004; Rockway and Giranda 2003). The ring is substituted for a basic moiety, either an amidine or a guanidine, which is essential to form a salt bridge with Asp189 of uPA.

The number of reports concerning peptides as urokinase inhibitors is limited. N-α-p-toluenesulphonyl-l-arginine methyl ester, ε-aminocaproic acid and its derivatives or natural leupeptin and its derivatives are known as inhibitors of urokinase (Rockway et al. 2002; Aoyagi et al. 1969). Glu-Gly-Arg-CH2Cl is a synthetic inhibitor which inhibits urokinase with Kᵢ value 5 µM and alkylates the active-site histidine of urokinase (Lijnen et al. 1987). But the most active peptide uPA inhibitor is phenethylsulfonyl-α-Ser-Ala-Arg-OH (Markowska et al. 2008) and the most active inhibitor of urokinase was H-D-Ser-Gly-Arg-OH with an IC₅₀ value of 24 M. The third series of compounds with urokinase and plasmin through a Surface Plasmon Resonance Imaging (SPRI).

Experimental

Reagents

Fmoc-Arg(Pbf)-OH (Fmoc = 9-fluorenylmethyloxy carbonyl, Pbf = pentamethyldihydrobenzofuran), chloranil, acetaldelyde, HOBt = 1-hydroxybenzotriazole, Fmoc-l-NVa-OH, Fmoc-l-Ala-OH, Fmoc-Gly-OH, TNBS = 2,4,6-trinitrobenzenesulfonic acid (1% solution in DMF), methanesulfonyl chloride, α-toluenesulfonyl chloride and 2,4,6-triisopro- pynylbenzenesulfonyl chloride were purchased from Fluka (Schnelldorf, Germany). Fmoc-l-Arg(t-Bu)-OH (t-Bu = t-buty1) and 2-chlorotryptyl chloride resin were purchased from Merck (Novabiochem, Darmstadt, Germany). TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, DIC = diisopropylcarbodiimide, piperidine, TBTU = tetrafluoroborate salt of the O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium tetrafluoroborate, NMP = 1-methyl-2-pyrrolidone and 1,5-diaminopentanetrityl resin were obtained from Iris Biotech GmbH (Marktretz, Germany). DCM = dichloromethane, DMF = dimethyl-formamide and methanol were the products of Chempur (Piekary Slaskie, Poland). DMF was used without further purification. DMF was distilled over ninhydrin and stored under molecular sieves 4A.

HPLC solvent acetonitrile was purchased from Merck (Darmstadt, Germany). Urokinase, trypsin, kallikrein 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). Ac-Leu-Leu-Arg-H, cysteamine hydrochloride, N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfuric acid) (HEPES), N-ethyl-N’- (3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (Steinheim, Germany). N-Hydroxysuccinimide amino acid in P2 (Markowska et al. 2012) but this class of peptides was not the inhibitors of urokinase.

The purpose of this paper was the synthesis and examination of the amidolytic activity of 17 novel peptides against urokinase, thrombin, trypsin, plasmin, t-PA and kallikrein. The peptides had the general X-SO₂-NH-D-Ser-AA-Arg-OH(NH-(CH₂)₅-NH₂) formula, where X = α-tolyl, p-tolyl, 2,4,6-triisopropylphenyl and AA = Ala, Gly, NVa (Table 1.). We tested the hemolytic activity of the peptides against porcine erythrocytes and the antitumor activity against the following human cancer cells: colorectal adenocarcinoma tumor DLD, standard MCF-7 and estrogen-independent MDA-MB-231. We also examined the interaction of some of the synthesised acids of tripeptides with urokinase and plasmin through a Surface Plasmon Resonance Imaging (SPRI).
NHS) was obtained from Aldrich (Munich, Germany). Thrombin and phosphate buffered saline (PBS) were purchased from Lubelska Wytwarńa Szczepionek (Lublin, Poland). t-PA was obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany). HBS-ES solution pH 7.4 (0.01 M HEPES, 0.15 M sodium chloride, 0.005 % Tween 20, 3 mM EDTA), acetic buffer pH 3.79–5.57, phosphate buffer pH 7.17–8.04, carbonate buffer pH 8.50–9.86 (Biomed, Lublin, Poland), photopolimer ELPEMER SD 2054, hydrophobic protective paint SD 2368 UV SG-DG (Peters, Kempen, Germany) were used as received. Aqueous solutions were prepared with MilliQ water (Simplicity® MILLIPORE).

Peptide Synthesis

The peptides shown in Table 1 were synthesized manually using the standard Fmoc-based strategy (Chan and White 2000). The Fmoc-Arg(Pbf)-OH was loaded on to the 2-chlorotriyl chloride resin with 2 M excess of DIPEA in DCM and in the case of 1,5-diaminopentane trityl resin using a molar ratio of amino acid/DIC/HOBt/resin 3:3:3:1. Fmoc deprotection steps were carried out with 20 % (v/v) piperidine in DMF/NMP 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. The sulfonyl chlorides were used as 5 M excess to resin and were dissolved in DCM with 10 M excess of DIPEA. The reactions were monitored with the Steward chloranil test (Vojkovski 1995) and with the TNBS test (Hancock and Battersby 1976). 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 precipitated and washed with cold diethyl ether, filtered, dissolved in water and lyophilized.

The Shimadzu LC-10A system (Shimadzu Europa GmbH, Duisburg, Germany) was used for analytical and semipreparatory HPLC (Phenomenex C18, Jupiter 90A, 4 micron, 250 × 10 mm; Phenomenex C18, Jupiter 300A, 5 micron, 250 × 4 mm; solvents: A, 0.1 % aqueous TFA; B, 0.1 % TFA in acetonitrile, gradient 0 % B to 100 % B 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 1).

Table 1  Analytical data of the synthesized compounds

| No. | Compound                              | Yield (%) | MW  | [M + H]^+  | Retention time (min) |
|-----|---------------------------------------|-----------|-----|------------|----------------------|
| 1   | PhCH₂SO₂-d-Ser-Ala-Arg-NH-(CH₂)₅-NH₂ | 47.3       | 570.7 | 571.8      | 13.36                |
| 2   | 2,4,6-TriiPrPhSO₂-d-Ser-Ala-Arg-NH-(CH₂)₅-NH₂ | 56.3       | 682.9 | 684.3      | 20.07                |
| 3   | CH₃PhSO₂-d-Ser-Ala-Arg-NH-(CH₂)₅-NH₂ | 54.6       | 494.6 | 495.3      | 13.99                |
| 4   | PhCH₂SO₂-d-Ser-Gly-Arg-NH-(CH₂)₅-NH₂ | 55.8       | 556.6 | 557.5      | 13.16                |
| 5   | 2,4,6-TriiPrPhSO₂-d-Ser-Gly-Arg-NH-(CH₂)₅-NH₂ | 49.7       | 668.8 | 669.6      | 21.23                |
| 6   | CH₃PhSO₂-d-Ser-Gly-Arg-NH-(CH₂)₅-NH₂ | 61.2       | 480.5 | 481.4      | 13.46                |
| 7   | PhCH₂SO₂-d-Ser-NVa-Arg-NH-(CH₂)₅-NH₂ | 56.7       | 598.7 | 599.9      | 14.08                |
| 8   | 2,4,6-TriiPrPhSO₂-d-Ser-NVa-Arg-NH-(CH₂)₅-NH₂ | 58.5       | 710.9 | 711.8      | 20.81                |
| 9   | CH₃PhSO₂-d-Ser-NVa-Arg-NH-(CH₂)₅-NH₂ | 59.7       | 522.6 | 523.5      | 14.94                |
| 10  | PhCH₂SO₂-d-Ser-Gly-Arg-OH             | 53.5       | 472.5 | 473.5      | 13.59                |
| 11  | 2,4,6-TriiPrPhSO₂-d-Ser-Gly-Arg-OH   | 56.7       | 558.7 | 558.8      | 21.24                |
| 12  | CH₃PhSO₂-d-Ser-Gly-Arg-OH            | 55.7       | 396.4 | 397.5      | 22.70                |
| 13  | PhCH₂SO₂-d-Ser-NVa-Arg-OH            | 54.6       | 514.6 | 515.7      | 15.33                |
| 14  | 2,4,6-TriiPrPhSO₂-d-Ser-NVa-Arg-OH   | 63.1       | 626.8 | 627.6      | 22.87                |
| 15  | CH₃PhSO₂-d-Ser-NVa-Arg-OH            | 52.1       | 438.5 | 439.4      | 16.17                |
| 16  | H-d-Ser-NVa-Arg-NH-(CH₂)₅-NH₂        | 62.4       | 444.5 | 445.9      | 8.57                 |
| 17  | H-d-Ser-Gly-Arg-NH-(CH₂)₅-NH₂        | 63.2       | 402.5 | 403.9      | 8.74                 |
(a) tris buffer—0.6 ml (pH 8.8),
enzyme: urokinase (50 U/ml),
synthetic substrate: S-2444 (0.1 ml, 3 mM);
(b) tris buffer—0.5 ml (pH 8.4),
enzyme: thrombin (1 U/ml),
synthetic substrate: S-2238 (0.2 ml, 0.75 mM);
(c) tris buffer—0.5 ml (pH 7.4),
enzyme: plasmin (0.4 U/ml),
synthetic substrate: S-2251 (0.2 ml, 3 mM);
(d) borane buffer—0.5 ml (pH 7.5),
enzyme: trypsin (0.4 U/ml),
synthetic substrate: Bzl-L-Arg-pNA.HCl (0.2 ml, 8 mM);
(e) tris buffer—0.6 ml (pH 9.0),
enzyme: kallikrein (3 U/ml),
synthetic substrate: S-2266 (0.1 ml, 7.5 mM);
(f) tris buffer—0.6 ml (pH 8.4),
enzyme: t-PA (1.67 mg/ml),
synthetic substrate: S-2288 (0.1 ml, 10 mM).

Buffer and 0.1 ml of enzyme solution was added to 0.2 ml of examined compound dissolved in 0.15 M NaCl (1–17) (as control 0.15 M NaCl). The mixture was incubated for 3 min at 37 °C, then the synthetic substrate was added. After 20 min of incubation, the reaction was stopped by adding 0.1 ml of 50 % acetic acid, and the absorbance of the released p-nitroaniline was measured at 405 nm (Spekol 1300, AnalyticJena). Every value represents the average of the triplicate determination. IC50 value 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. Ks was calculated from IC50 based on Cheng–Prusoff equation (Cheng and Prusoff 1973). The results are given in Table 2.

Our results were compared with the data obtained for Ac-Leu-Leu-Arg-H and Ac-Leu-Leu-Arg-COCHO (leupeptin—natural, microbial origin, inhibitor of proteinases and analog of leupeptin, respectively (Aoyagi et al. 1969)).

Antitumor Activity

Tissue Culture

All studies were performed on MCF-7, MDA-MB-231 and DLD cells lines were purchased from American Type Culture Collection (Rockville, USA). 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 % CO2 incubator.

Cytotoxicity Assay

The toxicity of the evaluated peptides was determined by the method of Plumb et al. (1989) in 10, 100, 250, 500 and 1,000 μM concentrations. 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 phosphate buffered saline (PBS). The cells were then incubated for 4 h in 2 ml of PBS with 50 ml of MIT (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 of synthesized peptides was calculated as percentage of non-viable cells and the IC50 value was estimated from logarithm curves as shown in Table 3.

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 1,000g for 10 min to remove plasma and the buffy coat. The various concentrations of peptides (100, 250, 500 and 1,000 μg/ml) were incubated with the erythrocyte suspension for 1 h at 37 °C (the final erythrocyte concentration was 5 % v/v). After the centrifugation (1,000 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 (TECAN, Salzburg, Austria) by measuring the absorbance at 414 nm. Zero hemolysis (blank), hemolysis with Ac-Leu-Leu-Arg-H as reference compound for synthesized 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 (%) = \( \frac{(Abs_{414\ nm\ in\ the\ peptide\ solution} - Abs_{414\ nm\ in\ 0.1\%\ Triton-X-100\ in\ PBS})}{Abs_{414\ nm\ in\ 0.1\%\ Triton-X-100\ in\ PBS}} \times 100. \)

SPRI Investigation

Chip Preparation

Gold chips were manufactured as described in previous papers (Gorodkiewicz 2009; Gorodkiewicz and Regulska 2010; Gorodkiewicz et al. 2011). The gold surface of the chip was covered with photopolymer and hydrophobic paint. 9 × 12 free gold surfaces were obtained. Using this chip,
nine different solutions can be simultaneously measured without mixing the tested solutions. Twelve single SPRI measurements can be performed from one solution.

**Inhibitor Immobilization**

Chips were rinsed with ethanol and water and dried under a stream of nitrogen. They were then immersed in 20 mM cysteamine ethanolic solutions for at least 2 h. The chips were then rinsed with ethanol and water and dried under a stream of nitrogen.

Among the synthesised compounds, four were selected for the examination of the interaction between the enzyme and the inhibitor. They were acids of peptides 10, 12, 13 and 14. Acids can be bound to a gold surface via cysteamine. A series of concentrations 0.002, 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 mM was used for each peptide. The concentration of the enzymes was constant 1 ng/ml. Inhibitor solution, activated with NHS (50 mM) and EDC (200 mM) was placed on the cysteamine-modified surface, and incubated at 37 °C for 1 h (Gorodkiewicz 2009; Gorodkiewicz and Regulska 2010; Gorodkiewicz et al. 2011). The chip was then treated with the urokinase or plasmin solution for 10 min, rinsed with...
HBS–ES buffer and dried. Finally, the SPRI measurement was performed. Each solution was put on two measuring fields (each consisting of 12 measuring points); thus 24 replicates were done in each case. The results are given in Figs. 1 and 2.

**SPRI Measurements**

SPRI measurements with an application of an enzyme array were performed as described in previous papers (Gorodkiewicz 2009; Gorodkiewicz and Regulska 2010; Gorodkiewicz et al. 2011). Briefly, the measurements were performed at a fixed angle of incident light and the reflectivity was simultaneously measured across an entire chip surface. The contrast values obtained for all pixels across a particular sample single spot were integrated. Thus the SPRI signal was integrated over the single spot area. Background correction was applied. NIH ImageJ version 1.42 software was used to evaluate the SPRI images in 2D form. The signal was measured twice on the basis of registered images, after immobilisation of enzymes and then after interaction inhibitors. The SPRI signal, which is proportional to coupled enzymes, was obtained from subtraction between the signal before and after interaction with enzymes for each spot separately.

**Results and Discussion**

Inhibition of the Serine Proteases

We expected that the use of the specific tripeptide sequence towards urokinase (D-Ser-AA-Arg) would cause some selectivity (Ke et al. 1997). According to the obtained results, only five compounds inhibited urokinase activity but none of them were selective. The most active inhibitor of u-PA was 10 PhCH$_2$SO$_2$-D-Ser-Gly-Arg-OH with $K_i$ value 5.4 µM. H-d-Ser-Gly-Arg-OH and H-d-Ser-Ala-Arg-OH were previously examined (Markowska et al. 2008) and they were the most active inhibitors of urokinase with IC$_{50}$ value of 1 mM and 2 mM respectively ($K_i$ = 90 µM and 180 µM, author’s unpublished data). In 2010 we synthesised N-sulfonlamide peptides with alanine as P2 (Markowska et al. 2010). α-Tolyl- and p-tolylsulfonylamides of the examined tripeptides were active towards urokinase. The compound 2,4,6-triisopropylphenylsulfonyl-D-Ser-Ala-Arg-OH was the most selective and we chose 2,4,6-triisopropylphenylsulfonyl fragment to the present research. In current work only compound 14 2,4,6-triPrPhSO$_2$-D-Ser-NVa-Arg-OH had some activity towards urokinase, but in this case it was not selective. Only amide of arginine 5 2,4,6-triPrPhSO$_2$-D-Ser-NVa-Arg-NH$_2$ and 2,4,6-triPrPhSO$_2$-D-Ser-NVa-Arg-NH$_2$$_3$-NH$_2$ were active urokinase inhibitors with $K_i$ value 44.6 µM.

All of the synthesised peptides were inhibitors of plasmin. The compound 1 PhCH$_2$SO$_2$-D-Ser-Ala-Arg-NH-(CH$_2$)$_3$-NH$_2$ was the most active plasmin inhibitor with $K_i$ value of 2 µM in this series of peptides. Compound 1 contains a fragment of pentamethylenediamine (cadaverine) as amide arginine residue in its structure which seems to have significant influence on the activity and selectivity. The moiety of cadaverine is the form of decarboxylated derivative of lysine. In literature, there are peptides with C-terminus lysine amide and also amide residue of lysine substituted with cadaverine, as inhibitors of plasmin (Markowska et al. 2007; Midura-Nowaczek et al. 2006). This kind of compounds was designed to be similar to natural substrate sequence hydrolysed by plasmin.

The most active inhibitors of thrombin in whole series of compounds were 10 PhCH$_2$SO$_2$-D-Ser-Gly-Arg-OH and 13
PhCH₂SO₂-D-Ser-NVa-Arg-OH with $K_i$ value 0.97 and 0.82 μM.

Antitumor Activity of Synthesised Peptides

The examined compounds 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 has low uPAR/uPA expressing and low plasminogen-binding, whereas MDA-MB-231 has high uPAR/uPA expressing and high plasminogen-binding (Stillfried et al. 2007). Thus, the influence of the synthesized compounds on the cytotoxic effect of MCF-7 cells could be insignificant.

The peptides 1–17 and the examined natural inhibitor of proteinases leupeptin were comparably cytotoxic to MDA-MB-231. The most interesting peptide was compound 10 with an IC₅₀ values of 5 μM for DLD cells, which was the most active inhibitor of urokinase in the amidolytic test.

Hemolytic Activity

The results indicated that the concentration up to 1,000 μg/ml of the synthesed peptides did not lyse porcine erythrocytes.

The Effect of Newly Synthesised Inhibitor Concentration on the SPRI Signals of Plasmin and Urokinase

The first aim of this investigation was the qualitative studies of the interaction between the potential inhibitor and the enzyme. The second aim was to find an optimal inhibitor concentration for the interaction with the enzymes.

The positive SPRI signal was obtained for urokinase after the interaction with inhibitors 10 and 14, as well as for plasmin with inhibitors 12 and 13. The obtained curves for compounds 10, 12, 13 and 14 were a Langmuir isotherm type, with the plateau of the signal above 0.02 mM. Leupeptin was used as a reference inhibitor for plasmin and urokinase. The newly synthesised inhibitors act in a similar way as leupeptin.

Conclusion

The most active compound towards urokinase was PhCH₂SO₂-D-Ser-Gly-Arg-OH with $K_i$ value 5.4 μM and the most active compound toward thrombin was PhCH₂SO₂-D-Ser-NVa-Arg-OH with $K_i$ value 0.82 μM. The peptides were nontoxic against porcine erythrocytes in vitro. PhCH₂SO₂-D-Ser-Gly-Arg-OH compound showed cytotoxic effect against DLD cell lines with IC₅₀ values of 5 μM. Some of the synthesised compounds bind to urokinase and plasmin in 0.05 mM concentration evaluated by the Surface Plasmon Resonance Imaging (SPRI) sensor.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

Aoyagi T, Miyata S, Nanbo M, Kojima F, Matsuzaki M, Ishizuka M, Takeuchi T, Umezawa H (1969) Biological activities of leupeptins. J Antibiot 11:558–568

Binder BR, Mihaly J (2008) The plasminogen activator inhibitor “paradox” in cancer. Immunol Lett 118:116–124

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 (IC₅₀) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

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 Design 10:39–49

Goodson RJ, Doyle MV, Kaufman SE, Rosenberg R (1994) High-affinity urokinase receptor antagonists identified with bacterio- phage peptide display. Proc Natl Acad Sci USA 91:7129–7133

Gorodkiewicz E (2009) Surface Plasmon Resonance Imaging sensor for cathepsin determination based on immobilized cystatin. Protein Pept Lett 16:1379–1385

Gorodkiewicz E, Regulska E (2010) SPR imaging biosensor for aspartyl cathepsins: sensor development and application for biological material. Protein Pept Lett 17:1148–1154

Gorodkiewicz E, Regulska E, Wojtulewski K (2011) Development of an SPR imaging biosensor for determination of cathepsin G in saliva and white blood cells. Microchim Acta 173:407–413

Hancock WS, Battersby JE (1976) A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzenesulphonic acid. Anal Biochem 71:260–264

Irignoy JF, Muñoz-Cánoves P, Montero L, Koziczk M, Nagamine Y (1999) The plasminogen activator system: biology and regulation. Cell Mol Life Sci 56:104–132

Kawada M, Umezawa K (1995) Suppression of in vitro invasion of human fibrosarcoma cells by a leupeptin analog inhibiting the urokinase–plasmin system. Biochem Biophys Res Commun 209:25–30

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

Klinghofer V, Stewart K, McGonigal T, Zhao X, Hulkower KI, Giranda VL (2001) Species specificity of a leupeptin analog inhibiting the urokinase–plasmin system. Biochem Biophys Res Commun 209:25–30

Klinghofer V, Stewart K, McGonigal T, Zhao X, Hulkower KI, Giranda VL (2001) Species specificity of amidine-based urokinase inhibitors. Biochemistry 40:9125–9131

Lijnen HR, Hoef B, Collen D (1987) Differential reactivity of Glu–Gly–Arg–CH₂Cl, a synthetic urokinase inhibitor, with single-chain and two-chain forms of urokinase-type plasminogen activator. Eur J Biochem 162:351–356

Longstaff C, Thelwell C (2005) Understanding the enzymology of fibrinolysis and improving thrombolytic therapy. FEBS Lett 579:3303–3309

Lynas JF, Martin SL, Walker B (2001) Synthesis and kinetic evaluation of peptide alpha-keto-beta-aldehyde-based inhibitors of trypsin-like serine proteases. J Pharm Pharmacol 53:473–480
Markowska A, Bruzgo I, Midura-Nowaczek K (2007) Low molecular peptides as potential inhibitors of plasmin. Acta Pol Pharm 64:355–358

Markowska A, Bruzgo I, Midura-Nowaczek K (2008) Effects of tripeptides on the amidolytic activities of urokinase, trombin, 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 Enzyme Inhib Med Chem 25:139–142

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

Markowska A, Bruzgo M, Surazyński A, Midura-Nowaczek K (2012) Tripeptides with C-terminal arginine as potential inhibitors of plasmin. J Enzyme Inhib Med Chem. doi: 10.3109/14756366.2011.651463

Midura-Nowaczek K, Lepietuszko I, Bruzgo I (2006) Synthesis of alkylamides of dipeptides as potential plasmin inhibitors. Acta Pol Pharm 63:33–37

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

Ossowski L, Aguirre-Ghiso JA (2000) Urokinase receptor and integrin partnership: coordination of signalling for cell adhesion, migration and growth. Curr Opin Cell Biol 12:613–620

Ploug M, Østergaard S, Gárdsvoll K, Kovalski K, Holst-Hansen C, Holm A, Ossowski L, Danø K (2001) Peptide-derived antagonists of the urokinase Receptor. Affinity maturation by combinatorial chemistry, identification of functional epitopes, and inhibitory effect on cancer cell intravasation. Biochemistry 40:12157–12168

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

Rockway TW, Giranda VL (2003) Inhibitors of the proteolytic activity of urokinase type plasminogen activator. Curr Pharm Design 9:1483–1498

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

Saino T, Someno T, Shin-Ichi I, Aoyagi T, Umezawa H (1988) Protease-inhibitory activities of leupeptin analogues. J Antibiot 41:220–225

Schweinitz A, Steimetz T, Banke IJ, Arlt MJE, Stürzebecher A, Schuster O, Geissler A, Giersiepen H, Zeslawska E, Jacob U, Krüger A, Stürzebecher 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 Met Rev 22:205–222

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

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

Vojkovski T (1995) Detection of secondary amines on solid phase. Pept Res 8:236–237