Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 4217–4220
Bioorganic & Medicinal Chemistry Letters

Design, synthesis and structure–activity relationship of a series of fragment analogues of antistasin (ATS) and ghilantens (GLS)

Dancho L. Danalev,* Lybomir T. Vezenkov and Boryana Grigorova

University of Chemical Technology and Metallurgy, Department of Organic Chemistry, 1756 Sofia, Bulgaria

Received 29 March 2005; revised 18 June 2005; accepted 24 June 2005
Available online 3 August 2005

Abstract—A series of low molecular weight peptide inhibitors of Factor Xa, fragment analogues of ATS and GLS, was designed and synthesized by the SPPS method. The new analogues included different basic amino acids in 109 position. In order to investigate the role of these factors, the newly synthesized peptides were tested for anticoagulant activity. To investigate the change in anticoagulant activity, new peptides were synthesized by replacement of the C-terminal COOH function with $CONH_2$. The biological activity of all compounds was measured in respect to APTT (activated partial thromboplastin time) and IC_{50} values (the concentrations for doubling APTT clotting times of human plasma) were determined.

1. Introduction

Antistasin (ATS), a 15 kDa anticoagulant protein isolated from salivary glands of the Mexican leech *Haementeria officinalis*, has been shown to be a potent inhibitor of Factor Xa in the blood coagulation cascade.[1] GLS are potent anticoagulant–antimetastatic proteins[2] produced in the salivary gland cells of the proboscis leech *Haementeria ghilianii*, a predatory annelid indigenous to the Amazon basin and surrounding region of South America.[3,4] The accompanying differences in IC_{50} values, as measured with the APTT blood coagulation assay, suggest that the C-terminus of ATS (amino acids 109–119) is an important determinant of inhibitory potency.[1] The differences between sequences 109–116 of rATS and natural GLS are in the position 109, where Arg residue is replaced by Lys, and in the position 115, where Ile residue is replaced by Val. In order to investigate the biological activity of sequence 109–116 of GLS, the role of hydrophilic amino acids Arg and Lys in 109 position, and the role of basic group in 109 position on the anticoagulant activity, a series of fragment analogues of ATS and GLS, including Arg, Lys and Orn in the N-terminus, was synthesized. On the assumption that replacement of COOH function with $CONH_2$ would lead to an increased stability of peptides against enzymatic hydrolysis and on the analogy of previously synthesized amides of analogues of isoform 3 of ATS, the amides of new peptides were also synthesized.[5]

2. Results and discussion

The synthesis of the peptides H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH, H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH, H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH, H-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH and their amides was realized by the SPPS method and Fmoc-strategy. The HBTU/DIPEA method was used for coupling of each amino acid. In the beginning, Wang resin was used to obtain analogues with free C-terminal COOH function. Some difficulties were met with during the synthesis of the peptides.

We did not succeed in obtaining Fmoc-Val-Pro-OH by use of Wang resin because of a side reaction, which leads to diketopiperasine.[6] Peptides containing the very difficult C-terminal Pro-Pro, Val-Pro and Tyr-Pro sequences could be obtained by Wang resin to an extent of less than 5%. In this case the Trityl resin can be used successfully.[7] For the synthesis of the target peptides with a free C-terminal COOH group, Trityl resin was used by us successfully.

Synthesis of peptides with $CONH_2$-function was realized by using Rink amide resin.

Keywords: Antistasin; Ghilantens; Factor Xa, Anticoagulant activity; Peptido mimetic.
* Corresponding author. Tel.: +359 2 8163418; fax: +359 2 8685488; e-mail: dantchoboy@abv.bg

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.06.078

The peptides obtained were studied for anticoagulant activity in respect to APTT (Tables 1–8) and IC_{50} values were determined (Table 9). The data for APTT in the tables refer to values above Ref. *t* 29.7 s.

The fragment (109–116) of natural GLS H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH has IC_{50} = 1118.0 nmol. Its analogue with $CONH_2$ function in C-terminus is 1.7 times more active (IC_{50} = 646.6 nmol). The most active are analogues obtained by replacement of Lys residue in 109 position with Arg. We synthesized the peptide H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH both by solid phase synthesis and in solution. It has 1.4 times higher activity than fragment (109–116) of natural GLS. The replacement of C-terminal COOH group by $CONH_2$ leads to a 4.3-fold increase in the activity towards the same (109–116) fragment, and a 2.5-fold increase towards its amide analogue. The replacement of Lys residue in the same position with Orn produces peptide H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH and its amide. They have IC_{50} values of 1156.0 and 705 nmol, respectively. The comparison of activities with fragment 109–116 of natural GLS shows that anticoagulant activity of both analogues are the same in the analogues with a C-terminal COOH group while it is slightly decreased in the analogues with $CONH_2$ function. The removal of Lys residue in 109 position resulted in analogue H-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH and its amide. They have IC_{50} values of 1562.1 and 2901.8 nmol. The lack of basic amino acid in 109 position resulted in a strongly decreased anticoagulant activity. A very interesting fact is that only in this case the analogue with C-terminal amide function has a 1.9-fold decreased activity in comparison to this analogue with COOH function. One of the most frequently used anticoagulant drugs in medical practice is Phenylin. It has an IC_{50} value of about 10^{-6} mol, which means it is about 1000-fold less active than the newly synthesized analogues, except for analogues without basic amino acid in 109 position, which have the commensurable activity with Phenylin.

Table 1. Anticoagulant activity of the compound H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH

Concentration (mol·10^{-9})	APTT (s)
41.89	No activity
272.29	3.8
418.91	5.5
523.64	15.6
802.24	62.4

Table 2. Anticoagulant activity of the compound H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH

Concentration (mol·10^{-9})	APTT (s)
290.00	6.7
894.00	21.6
1043.42	24.1
1117.95	30.0
1192.00	50.9
2235.90	122.8

Table 3. Anticoagulant activity of the compound H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH

Concentration (mol·10^{-9})	APTT (s)
771.00	22.2
1156.50	28.5

Table 4. Anticoagulant activity of the compound H-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH

Concentration (mol·10^{-9})	APTT (s)
1338.96	22.1
1562.12	31.0
1785.28	47.1

Table 5. Anticoagulant activity of the compound H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2

Concentration (mol·10^{-9})	APTT (s)
200.50	10.0
260.70	29.9
300.80	45.0

Table 6. Anticoagulant activity of the compound H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2

Concentration (mol·10^{-9})	APTT (s)
211.00	8.9
646.61	28.9
1077.70	57.9

Table 7. Anticoagulant activity of the compound H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2

Concentration (mol·10^{-9})	APTT (s)
528.94	0.5
705.25	29.3
881.56	53.3

Table 8. Anticoagulant activity of the compound H-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2

Concentration (mol·10^{-9})	APTT (s)
906.80	8.8
1813.59	18.6
2266.99	18.8
2720.39	25.5
2901.75	29.8
2992.43	32.4

Table 9. IC_{50} values of newly synthesized peptides

Peptide	IC_{50} (mol·10^{-9})
H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH	802.2
H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH	1118.0
H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH	1156.6
H-Pro-Lys-Arg-Lys-Leu-Val-Pro-OH	1562.1
H-Arg-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2	260.7
H-Lys-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2	646.6
H-Orn-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2	705.3
H-Pro-Lys-Arg-Lys-Leu-Val-Pro-NH_2	2901.8

3. Experimental

The amino acid used for synthesis of the target peptides were protected as: Fmoc-Arg(Pbf); Fmoc-Lys(Boc) and Fmoc-Orn(Boc).

3.1. Chemical part

The structures of the newly synthesized peptides were analysed and proved by mass spectrometry. The purity of the compounds was monitored on the RP-HPLC by isocratic elution by 70%AcCN/30%NaH_2PO_4–Na_2H-PO_4 buffer pH 7, ODS column, 1 mL/min and $\lambda = 220$ nm.

3.2. Pharmacological part

The APTT in human poor platelet plasma (PPP) was measured on the Amelung coagulometer KC4A apparatus by incubation of 450 μL citrated PPP at different concentrations of the peptides.

PPP (pure platelet plasma): Blood samples were collected in one-tenth volume of 3.8% sodium citrate by venipuncture from healthy volunteers in vacuumtainers with collection tubes. Whole blood was centrifuged at 300*g* for 5 min to separate the platelet-rich plasma. The remainder was centrifuged at 3400*g* for 10 min and PPP was collected.

3.2.1. General procedure for attachment of amino acid to Trityl resin. A total of 0.6–1.2 equiv relative to the resin carboxylic acid and DIPEA (4 equiv to carboxylic acid) were dissolved in dry DCM (10 ml per gram of resin). The reaction mixture was stirred for 30–120 min and at the end of reaction, it was washed by 3× DCM/CH_3OH/DIPEA (17:2:1); 3× DCM; 3× DMF; 3× DCM. The obtained Fmoc-amino acid resin was dried in vacuum over $CaCl_2$.

3.2.2. General procedure for attachment of amino acid to Rink amide resin. The Rink amide resin was allowed to pre-swell with DCM for 1 h and then washed thoroughly with DMF. The resin was then treated with 20% piperidin in DMF for 20 min to remove the Fmoc-group and then washed with 3× DMF and 3× DCM. The amino acid (4 equiv relative to resin loading) and HBTU (4 equiv relative to resin loading) were dissolved in DMF and DIPEA (5 equiv relative to resin loading) was added. The reaction mixture was left for 10 min and then added to the resin mixture in DMF. After 2 h the Kaiser test was performed.

3.2.3. General procedure for coupling of amino acids by HBTU/DIEA method. The Fmoc-amino acid (4 equiv relative to resin loading), HBTU (4 equiv relative to resin loading) and DIPEA (4 equiv relative to resin loading) were dissolved in DMF. The reaction mixture was stirred for 10 min and added to the peptide–resin solution in DMF. The reaction mixture was left for 2 h. The Kaiser test was performed at the end of the reaction time.

3.2.4. Preparing the peptide–resin for cleavage. The peptide–resin obtained was washed by 3× DMF; 3× DCM; 3× *i*-propanol and 3× ether to shrink the resin. The peptide–resin was then dried under high vacuum for 4 h over NaOH.

3.2.5. TFA cleavage and deprotection for peptide–Trityl resin. The dry peptide–resin was placed in a flask and TFA/DCM (1:1) were added for 2 h. At the end of the reaction time, the peptide was collected by filtration and the removed resin was washed by 3× TFA and 3× DCM. The filtrates were collected and evaporated to dryness.

3.2.6. TFA cleavage and deprotection for peptide–Rink amide resin. The dry peptide–resin was placed in a flask and TFA/TIS/H_2O (95:2.5:2.5) were added for 5 h. At the end of the reaction time, the peptide was collected by filtration and the removed resin was washed by 3× TFA and 3× DCM. The filtrates were collected and evaporated to dryness.

4. Conclusions

The following conclusions may be drawn from our investigation on the anticoagulant activity of a series of new analogues of rATS (109–116) and GLS (109–116):

1. Removal of basic amino acid in 109 position leads to a significant decrease of anticoagulant activity.
2. Availability of Lys residue exactly in 109 position is not significant for anticoagulant activity.
3. Increasing basic properties of amino acid in 109 position results in increased anticoagulant activity.
4. Replacement of Arg residue in 109 position by Lys and Orn leads to a comparable decrease of anticoagulant activity. This result shows that Arg residue has an essential role on the anticoagulant activity and minimal differences between Lys and Orn structures do not affect the anticoagulant activity.
5. The creation of the amide function in the C-terminus of newly synthesized analogues leads to a two-fold increase of anticoagulant activity with the exception of analogues without basic amino acid in 109 position.

Acknowledgments

This work was supported by the Scientific Research Fund, Ministry of Education and Science, Contract 783.

References and notes

1. Mao, S. S.; Huang, J.; Neeper, M. E.; Shafer, J. A. *Thromb. Haemostasis* **1993**, *69*, 1046.
2. Brankmp, R. G.; Blankenship, D. T.; Sunkara, P. S.; Cardin, A. D. *J. Lab. Clin. Med.* **1990**.
3. de Filipp, F. *Med. Accad. Sci. Torino* **1849**, *10*, 1.
4. Sawyer, R. T. *Yb. Am. Phil. Soc.* **1978**, 212.

5. Danalev, D.; Vezenkov, L.; Grigorova, B.. *J. Peptide Sci.* **2004**, *10*, 17.
6. Fischer, P. M. *J. Peptide Sci.* **2003**, *9*, 9.
7. Gruebler, G.; Zimmermann, H., et al. Solide phase synthesis of C-terminal proline peptides using an anchor system suppressing diketopiperasine formation. *An Innovation and Perspective in Solid Phase Synthesis. Peptides, Proteins and Nucleic Acids. Biological and Biomedical Applications*; Mayflower Worldwide: Birmingham, 1994, pp 517–520.