Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 733–736

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

The Design of Phenylglycine Containing Benzamidine Carboxamides as Potent and Selective Inhibitors of Factor Xa

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Received 28 April 2000; revised 29 November 2000; accepted 16 January 2001

Abstract—Factor Xa, a critical serine protease in the blood coagulation cascade, has become a target for inhibition as a strategy for the invention of novel anti-thrombotic agents. Here we describe the development of phenylglycine containing benzamidine carboxamides as novel, potent and selective inhibitors of factor Xa.

Serine protease factor Xa is central to the blood coagulation cascade, responsible for the generation of thrombin by both intrinsic and extrinsic pathways. This is achieved by combination of factor Xa, Ca^{2+} and phospholipids, forming the prothrombinase complex, which generates active thrombin by proteolysis of the zymogen prothrombin. Thrombin is responsible for the activation of platelets, the feedback activation of other coagulation factors and the conversion of fibrinogen to fibrin which crosslinks and stabilises the aggregating platelet plug. Following the extensive investigation of inhibitors of thrombin as potential antithrombotic agents,[1] small molecule inhibitors of factor Xa are increasingly being reported as potential anti-thrombotic agents.[2,3]

Benzamidine-3-carboxamide **1**, K_i 12 μM against factor Xa, was selected from a small library of compounds designed as potential factor Xa inhibitors using PRO_SELECT, a module[4] within our proprietary modelling software suite Prometheus™. Examination of a model of **1** docked into the active site of factor Xa,[5] suggested that further elaboration by replacement of the central glycine with lipophilic D-amino acids might result in an additional interaction with the lipophilic 'disulphide' pocket adjacent to S1, comprising residues Gln192, Cys191, Cys220 and Gly218. Accordingly a small library of compounds was generated and synthesised using primarily lipophilic D-amino acids. Additional non-preferred L-amino acids were also included as control molecules. Thus, bis-1,4-aminomethylcyclohexane, immobilised on chlorotrityl resin, was condensed with Fmoc-amino acids using TBTU/DIPEA.[6] Fmoc deprotection with piperidine and condensation with benzamidine-3-carboxylic acid using HOBt/DIPCI[6] gave a set of 32 compounds.[7] Table 1 shows binding data[8] for some of these compounds (**1**–**8**) against factor Xa and the structurally related enzyme trypsin (used as a measure of selectivity). The results for compounds **2**–**7** show a marked preference for compounds derived from D-amino acids as suggested by the model with, for the most part, some small selectivity for factor Xa over trypsin. Compound **8**, a racemic mixture,[9] showed the greatest affinity for factor Xa. A second library of 20 aryl glycines and analogues was designed and synthesised as before, selected results are also shown in Table 1 compounds **9**–**15**. In this second library for the most part racemic starting amino acids were used, the exception being D- and L-cyclohexylglycine.

Compounds **14** and **15** derived from L- and D-cyclohexylglycine, respectively, show the same preference for D-amino acid derived compounds as previously

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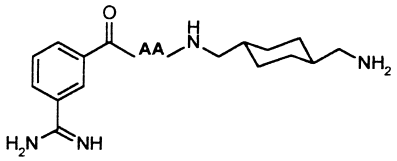
0960-894X/01/$ - see front matter
PII: S0960-894X(01)00042-7

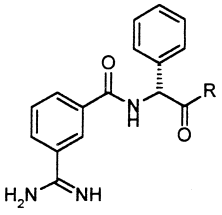
Table 1. Replacement of glycine in compound **1**

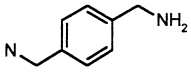
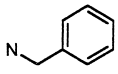
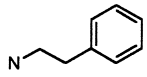
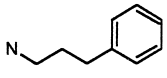
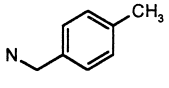
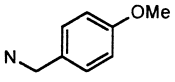


	Amino acid AA	FXa K_i (μM)	Trypsin K_i (μM)
1	Gly	12.0	23.0
2	L-Val	38.0	12.6
3	D-Val	6.6	16.2
4	L-Met	10.5	7.9
5	D-Met	2.3	10.0
6	L-Phe	33.9	12.6
7	D-Phe	25.7	17.4
8	D,L-Phg[a]	0.3	4.5
9	D,L-3-Cl-Phg	0.3	3.9
10	D,L-4-Cl-Phg	2.5	6.0
11	D,L-3-Cl-Phg	0.3	5.1
12	D,L-4-Ph-Phg	0.8	1.1
13	D,L-1-Nag[b]	0.1	2.1
14	L-Chg[b]	7.8	3.6
15	D-Chg[b]	0.5	8.5

[a]Compounds prepared from D- and L-phenyl glycine suffered complete racemisation using our initial coupling conditions in ref 9.
[b]Thg, thienylglycine, Nag, naphthylglycine, Chg, cyclohexylglycine.

Table 2. Primary amine–S4 library



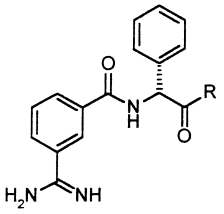
	R	FXa K_i (μM)	Trypsin K_i (μM)	Thrombin
8		0.275	5.6	6.5
16		0.500	4.1	26
17		0.910	5.4	8.5
18		0.850	3.3	3.1
19		0.145	2.2	NA[a]
20		0.042	4.9	1.9
21		0.028	5.5	2.3

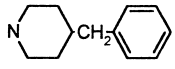
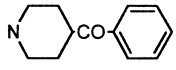
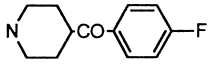
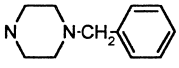
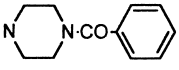
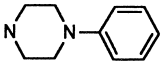
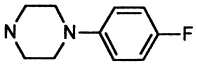
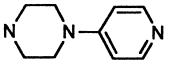
[a]NA denotes data not available.

noted. Molecular modelling of the two phenylglycine enantiomers of **8** in the active site of factor Xa shows the benzamidine in the S1 pocket with a bidentate interaction with Asp189 whilst the cyclohexylaminomethyl moiety projects towards the S4 pocket bordered by Phe174, Trp215 and Tyr99. The benzene ring of D-phenylglycine is accommodated in the lipophilic 'disulphide' pocket described above. This interaction is not possible with the enantiomer derived from L-phenylglycine. Despite the greater activity of compound **13**, derived from D,L-1-naphthylglycine, further studies were carried out using the more accessible and less lipophilic D-phenylglycine.

To explore the S4 region of factor Xa, several diverse compound libraries were designed and synthesised using coupling conditions which gave much reduced racemisation of the D-phenylglycine[10] (typically $< 5\%$ racemisation by HPLC examination of products derived from chiral amines). Tables 2 and 3 show the activities of some of these compounds (selected from a total of approximately 200) assayed against factor Xa, trypsin and thrombin (as a measure of selectivity against related

Table 3. Secondary amine–S4 library



	R	FXa K_i (μM)	Trypsin K_i (μM)	Thrombin K_i (μM)
22		0.030	0.65	1.35
23		0.013	0.32	1.26
24		0.010	0.54	1.18
25		0.120	2.20	7.94
26		0.282	0.46	7.94
27		0.016	0.34	1.74
28		0.015	1.26	3.80
29		0.013	0.56	17.80

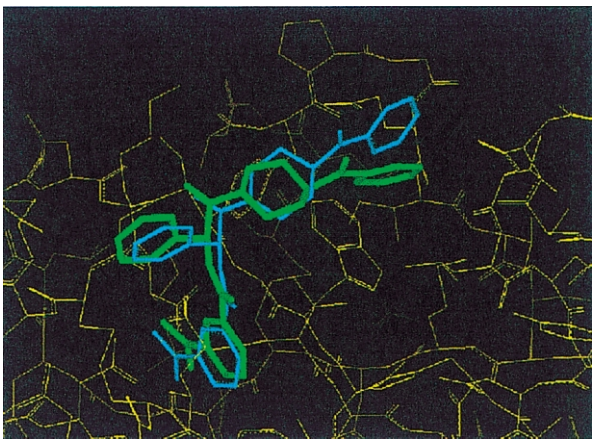


Figure 1. Compound **24** as modelled in the active site of fXa (blue) compared to the X-ray structure of **24** crystallised in trypsin (green).

enzymes). It may be seen that replacement of the cyclohexane ring of **8** with a benzene ring **16** gave only a modest loss of activity against factor Xa (**8** 0.27 μM vs **16** 0.5 μM) with a significant gain in selectivity against thrombin (6.5 vs 26.0 μM).

Deletion of the terminal methylene amino group, **17**, gave a loss in activity (0.91 μM) which was recovered by a two carbon chain extension, **19** (0.145 μM), where the benzene ring can make a closer lipophilic interaction in S4. (Further chain extension was detrimental to activity.) Substitution in the 4-position of the benzene ring of compound **17** gave **20** (0.042 μM) and **21** (0.028 μM) showing a 21- and 32-fold increase in activity against factor Xa compared to compound **17** with no increase in activity against trypsin.

Libraries designed using more constrained secondary amines, for example, piperidines and piperazines, were particularly effective, yielding compounds in the low nanomolar range albeit with some increased activity towards trypsin and thrombin. Thus, substituted piperidines **22**, **23** and **24**, more rigid examples of the extended alkyl amine **18** and **19**, showed improved activity towards factor Xa, the larger gains seen with a carbonyl linkage between the piperidine and the benzene ring.

This was not seen in the piperazine series where the resulting amide of compound **26** would favour a non-optimal conformation for the benzene ring. Here, the best activity against factor Xa was found with the benzene ring directly attached to the piperazine nitrogen as in compounds **27**, **28** and **29**.

Support for our modelled binding mode was provided by crystallographic studies using the surrogate enzyme trypsin which is structurally similar to factor Xa. Figure 1 shows compound **23** as modelled in the active site of factor Xa (blue) as compared to the X-ray structure of **23** crystallised in trypsin (green). The benzamidine is sited in the S1 pocket forming a bidentate interaction with Asp189 whilst the benzene ring of the D-phenylglycine makes the proposed lipophilic interaction in the 'disulphide' pocket which is also accessible in trypsin. The ligand makes two other hydrogen bonds with trypsin, the first with Gly216 through the 3-benzamide NH and the second with Gly218 through the phenylglycine carbonyl. The small differences observed in the conformation of the benzoylpiperidine possibly reflect the more open nature of the structure of trypsin in the S4 region.

Conclusions

Using PRO_SELECT, a number of highly focused libraries of compounds have been designed and synthesised giving rapid access to a series of potent and selective inhibitors of factor Xa. Key to the potency of these compounds is the lipophilic interaction between the phenylglycine residue and the lipophilic 'disulphide' pocket comprising Gln192, Cys191, Cys220 and Gly218. This is the first example reported of the use of modelling of this interaction in the design of inhibitors for factor Xa.

We are currently researching additional novel series of orally available inhibitors of factor Xa design using PRO_SELECT.

References

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2. Sinha, U. *Exp. Opin. Invest. Drugs* **1999**, *8*, 567. Walenga, J. M.; Jeske, W. P.; Hoppensteadt, D.; Kaiser, B. *Current Opinion in Cardiovascular, Pulmonary & Renal Investigational Drugs* **1999**, *1*, 13. Bing-Yan Scarborough, Z. *R. M, Current Opinion in Cardiovascular, Pulmonary & Renal Investigational Drugs* **1999**, *1*, 63.
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4. Murray, C. W.; Clark, D. E.; Auton, T. R.; Firth, M. A.; Li, J.; Sykes, R. A.; Waszkowycz, B.; Westhead, D. R.; Young, S. C. *J. Comput.-Aided Mol. Des.* **1997**, *11*, 193. For additional information on Protherics software suite see www.protherics.com.
5. Initial work in this series of compounds was carried out using the crystal structure as described by Padmanabhan, K. P.; Tulinsky, A.; Park, C. H.; Bode, W.; Huber, R.; Blankenship, D. T.; Cardin, A. D.; Kiesel, W. *J. Mol. Biol.* **1993** *232*, 947.

6. The following abbreviations have been used. TBTU: 2-(1*H*-benzotriazole-1-yl)-1,3,3-tetramethyluronium tetrafluoroborate. DIPEA: diisopropylethylamine. HOBt: 1-hydroxybenzotriazole. DIPCI: diisopropylcarbodiimide. EDC: 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide·HCl. HOAt: 1-hydroxy-7-azabenzotriazole.

7. Satisfactory spectral data was obtained for all compounds.

8. K_i values were determined (minimum $n=2$) according to the method of Tapparelli. Compounds were dissolved in Me_2SO, and diluted into assay buffer: 100 mM phosphate buffer, 0.1% bovine serum albumin, pH 7.4. Enzymes were supplied by Calbiochem, San Diego, CA and substrates by Pentapharm, Basel, Switzerland. The final concentrations of enzyme and substrate were: 100 pM human fXa/125 μM Pefachrome-FXA (K_m 72 μM); 1 nM human thrombin/125 μM Pefachrome-TH (K_m 5.4 μM); 1 nM human trypsin/125 μM Pefachrome-TRY (K_m 47 μM) (Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Zurini, M.; Claeson, G.; Scully, M. F.; Stone, S. R. *J. Biol. Chem.* **1993**, *268*, 4734). Increasing optical density at 405 nM caused by substrate hydrolysis was measured on a Labsystems Ascent Microplate reader (Labsystems, Cambridge, UK). K_m and K_i were calculated using SAS PROC NLIN (SAS Institute, Cary, NC, USA, Release 6.11).

9. Initial coupling conditions used on solid phase gave complete racemisation of D- and L-phenylglycines, the results obtained are reported as racemic D,L mixtures. Accordingly milder coupling conditions were developed for solution-phase chemistry of later compounds.

10. General method of solution-phase synthesis of library compounds: A solution of EDC (1.1 equiv) and HOAt (1.3 equiv) in 1:1 dichloromethane/DMF was stirred at ~5 °C for 10 min. Boc-phenylglycine (1.0 equiv) was added and stirring continued for 10 min before adding to the amine (1.1 equiv). After 2 h, during which time the mixture was allowed to warm to room temperature, the solution was partitioned between water and ethyl acetate. The product was isolated by evaporation of solvent and purified as necessary using flash chromatography. (HPLC examination of products derived from chiral amines typically showed < 5% of the diastereomeric product.) Removal of the Boc protection was carried out using 1:1 dichloromethane/trifluoroacetic acid and the products isolated as free bases by partitioning between ethyl acetate and sodium bicarbonate solution. The final coupling with 3-amidino-benzoic acid was carried out as follows: HOBt (1 mmol, 136 mg) in DMF (4 mL) was stirred in an ice bath and treated with DIPCI (1 mmol, 160 μL). After 10 min, 3-amidino-benzoic acid TFA salt (1 mmol, 278 mg) was added and stirring continued for a further 10 min at room temperature. This mixture was then added to the D-phenylglycine 'amide' (1 mmol) and stirred overnight. The crude product was then dissolved in water/acetonitrile (20 mL), filtered and purified by preparative HPLC.