European Journal of Medicinal Chemistry 98 (2015) 49–53

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

(±) *cis*-bisamido epoxides: A novel series of potent FXIII-A inhibitors

Craig A. Avery [a], Richard J. Pease [b], Kerrie Smith [b], May Boothby [b], Helen M. Buckley [b], Peter J. Grant [b], Colin W.G. Fishwick [a, *]

[a] School of Chemistry, University of Leeds, Leeds LS2 9JT, UK
[b] Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds LS2 9JT, UK

ARTICLE INFO

Article history:
Received 9 December 2014
Received in revised form
11 May 2015
Accepted 13 May 2015
Available online 16 May 2015

Keywords:
Thrombosis
FXIII-A
Bis-amido epoxides molecular modelling

ABSTRACT

A novel class of potent FXIII-A inhibitors containing a (±) *cis*-bisamido epoxide pharmacophore is described. The compounds display highly potent inhibition of FXIII-A (IC_{50} = 5–500 nM) in an *in vitro* assay. In contrast to other types of previously described covalent transglutaminase inhibitors, the bis-amido epoxides exhibited no measurable reactivity with glutathione, therefore possibly rendering this class of compounds suitable for future *in vivo* investigations. Additionally, the compounds show selective inhibition for FXIII-A against the cysteine protease, cathepsin S although they proved to have similar potency with a closely related transglutaminase, TGII, to that observed for FXIII-A.

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1. Introduction

FXIII is the final enzyme involved in the blood clotting process, its major function being to introduce covalent cross links between adjacent fibrin strands in a developing blood clot and covalently attaching inhibitors of fibrinolysis, thus imparting a vastly increased stability and lifetime to the clot [1,2]. Plasma FXIII circulates as a tetramer composed of two A domains and two B domains. Cellular FXIII however is composed only of the two A chains, suggesting the B domains are necessary for transportation of the catalytic A regions in blood plasma. Several crystal structures of human zymogen FXIII have been solved and consistently reveal the presence of a C314, H373, D396 triad [3]. Zymogen FXIII has no transglutaminase activity as the catalytic triad is buried deep in the core domain of the enzyme surrounded by two barrel domains, which block the entrance to the active site of the enzyme [4]. Conversion into the active transglutaminase (FXIII-A) is mediated by thrombin and Ca^{2+} and involves a large conformational change, which removes the barrel domains from the core region thus allowing the substrates access to the active site [5].

Factor XIII-A inhibitors do not prevent fibrin clot formation; however they do inhibit the production of cross-linked, polymeric clots. It has thus been proposed that selective inhibition of FXIII-A would provide a mild and safe therapeutic approach for conditions such as thrombosis, atherosclerosis and coronary heart disease [6]. Numerous *in vitro* [6] and several *in vivo* [7,8] trauma induced thrombosis models have provided strong evidence that this approach could provide a useful adjunct to current treatment protocols.

There have only been a small number of non-peptidic FXIII-A inhibitors identified to date. Two notable examples are the natural products alutacenoic acids A and B [9], and the imidazo[1,2-d][1,2,4]-thiadiazoles [10], such as compound **1**. FXIII-A is inhibited irreversibly by these molecules *via* attack of the active site cysteine, C314, on electrophilic moieties present in the inhibitors. The mechanism of FXIII-A-catalysed fibrin cross-linking suggests that the presence of a competing amine substrate will impede the formation of fibrin cross-links. Lorand et al. thus established that primary amines that can serve as substrates for the enzyme will become incorporated into fibrin [11]. The same result can be obtained *via* the use of pseudo-acceptor moieties, using glutamine isosteres [12]. Several examples of these inhibitors have been discovered for FXIII-A. In addition, a number of FXIII-A inhibitors e.g. the peptide tridegin [13], ZG-1400 [14] and natural product cerulenin **2** [15], were discovered through screening of natural product libraries (Fig. 1). The inhibitors of FXIII-A currently reported are severely lacking in many of the key criteria for successful *in vivo* application and thus none have been investigated as potential therapeutics. Therefore, there is a need to identify potent and selective small molecule inhibitors for this enzyme that may

* Corresponding author.
E-mail address: C.W.G.Fishwick@leeds.ac.uk (C.W.G. Fishwick).

http://dx.doi.org/10.1016/j.ejmech.2015.05.019
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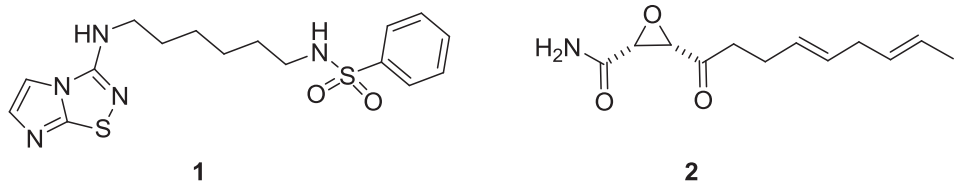


Fig. 1. Examples of small molecule inhibitors of FXIII-A. Both compounds inhibit FXIII-A *via* reaction with the active site cysteine C314.

offer potential for therapeutic lead development. Here we report the identification of a new class of FXIII-A inhibitors which exhibit excellent inhibitory potency and hold potential for future development for thrombosis therapy.

2. Results and discussion

Having studied a number of electrophilic moieties as covalent 'warheads' in the course of our FXIII-A research, it was apparent that many of them were susceptible to reaction with endogenous nucleophiles such as glutathione, which may compromise their potential for *in vivo* application. Thus intracellular glutathione is present at millimolar concentrations, but even if it were desirable to selectively inhibit plasma FXIII-A with membrane impermeant reagents, glutathione is present at micromolar concentrations in plasma. However, cerulenin **2** which contains an epoxide moiety as its electrophilic centre displays moderate inhibition of FXIII-A and is unaffected by a large excess of glutathione. In particular, performing the assay in the presence of 1 mM of reduced GSH affected the IC_{50} only marginally, decreasing it from 4 μM to 18 μM (Fig. 2).

Cerulenin has previously been shown to inhibit the fatty acid synthase enzyme (FAS) complex, which involves covalent bond formation *via* attack of an active site cysteine thiol on the epoxide moiety within the natural product with subsequent ring opening [16]. In light of the interesting inhibitory properties displayed by cerulenin in the presence of FXIII-A, we were keen to explore the possible binding mode of this natural product with a view to designing variants that would be amenable to synthetic manipulation in order to enable SAR studies. Therefore, assuming that a similar cysteine-based epoxide opening mechanism to that observed with FAS was present, molecular modelling was performed in order to deduce a likely binding pose for the pre-covalent attack complex of cerulenin within the FXIII-A active site and to also

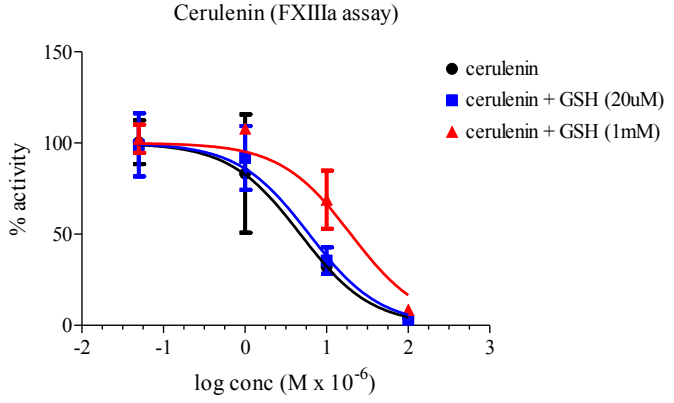


Fig. 2. Dose response curve for cerulenin induced FXIII-A inhibition. Cerulenin (black curve) inhibits with an IC_{50} of 4 μM. GSH addition at 20 μM (blue curve) and 1 mM (red curve) are also shown. The IC_{50} is only marginally changed by the addition of GSH as indicated by the close overlap of the dose response curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aid in the design of analogues. For these in silico studies, in the absence of a crystal structure of the activated form of the enzyme, a model of FXIII-A was created from the published zymogen crystal structure (1GGT.pdb) [4], by manually deleting the 'blocking' barrel domains (involving deletion of 229 residues between W500 – P729 inclusive) to reveal the active site. Cerulenin was then manually positioned into this active site model of FXIII-A. Two constraints were placed on the positioning of the epoxide moiety; firstly the epoxide oxygen was placed in close proximity to the backbone N–H of C314 as a primary 'oxyanion hole' residue [3]. Secondly, the carbon atom immediately adjacent to the primary amide unit within cerulenin was placed within close proximity to the thiol moiety of C314, thus allowing the attack/ring opening of the epoxide moiety to occur. Following this process, the complex was minimised whilst freezing the protein atoms and including a constraint such that the distance between C314 and the electrophilic carbon of the epoxide was maintained at a distance of 2.4 Å to simulate the necessary positioning of the thiol group immediately prior to epoxide ring opening. This process led to the identification of a binding mode very similar to that observed between cerulenin and FAS, which has been previously characterised using X-ray crystallography [16]. In particular, the FAS binding mode of cerulenin reveals that inhibition involves attack by the active site cysteine thiol on the epoxide ring whilst initially bound to the enzyme as a non-covalent complex. After reaction, the resulting oxyanion is stabilised *via* H-bonding with backbone N–H's within the 'oxyanion hole' of the enzyme. The binding mode predicted herein supports a similar mechanism for FXIII-A inhibition involving attack by C314 at the 2-position (bearing the primary carboxamide) on the epoxide ring of cerulenin. Furthermore, the modelling suggested that replacing the 3-keto unit within cerulenin with an amide moiety was predicted to enhance binding to FXIII-A in the initial complex *via* formation of an additional H-bond from this amide N–H to the backbone carbonyl of Y372. Additionally, replacement of the alkenyl chain in cerulenin with an aromatic ring was predicted to result in a possible π-stacking interaction with Y372 and, again, potentially enhancing binding of the inhibitor within the 'pre-attack' complex (Fig. 3).

In light of the predictions from the modelling studies, it was desirable to prepare a small series of potential FXIII-A inhibitors based upon a *cis*-bisamido epoxide motif. The modelling had indicated that the absolute stereochemistry within cerulenin (2*R*, 3*S*) and, by analogy, within the designed bis-amido epoxides, as shown above (Fig. 3) was important and that stereoisomeric epoxides would bind less efficiently. However, in order to expedite synthesis and biological evaluation of molecules corresponding to the designed inhibitors, it was decided to prepare the inhibitors as racemates.

Initially, synthesis of compounds **7** and **8** was achieved through the intermediate succinimides **5** and **6**, following ring opening with ammonia. Succinamides **5** and **6** were readily obtained in two steps from diacid **3** (Scheme 1).

Unfortunately this approach proved to have limited scope and was therefore discontinued in favour of an alternative approach involving amide coupling to amido acid **9**. Thus, anhydride **4** was

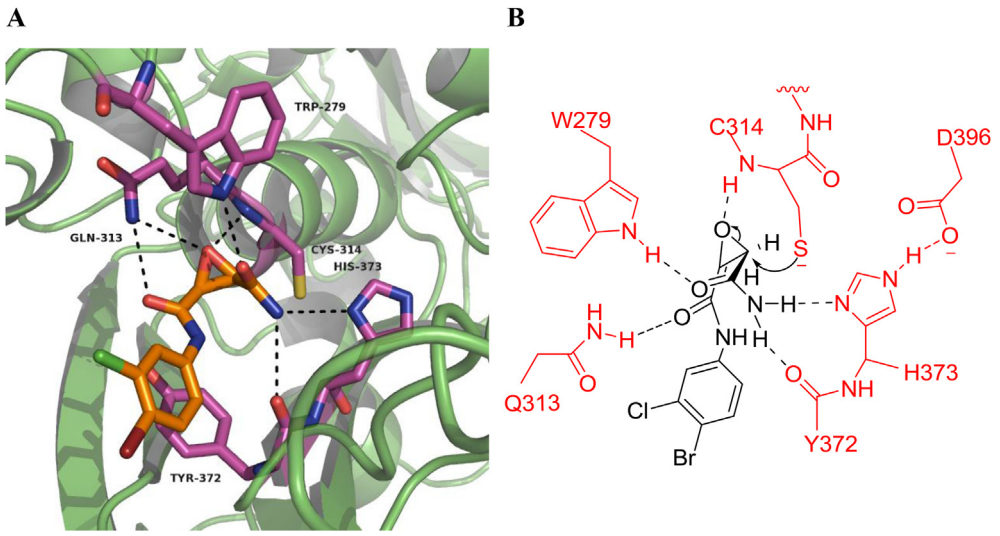
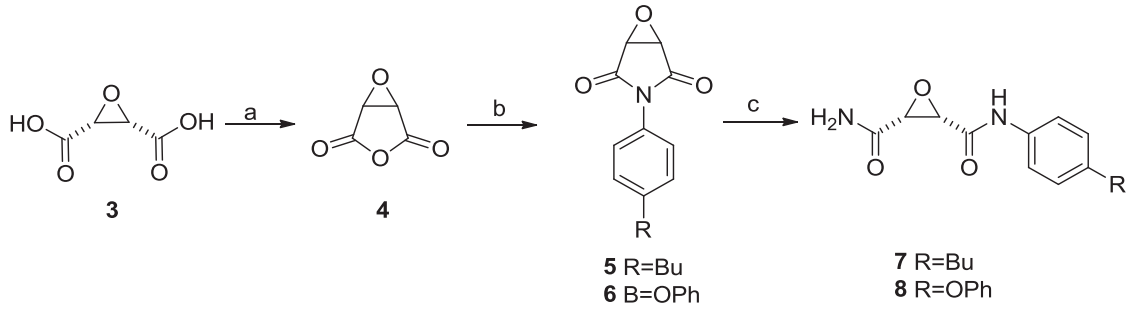


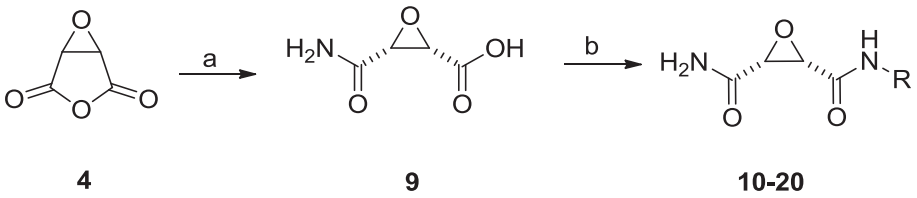
Fig. 3. (A) Predicted binding mode of epoxide **16** within the FXIII-A active site. (B) Schematic diagram of the predicted polar contacts between epoxide **16** and FXIII-A and the proposed mechanism of inhibition by epoxide **16**.



Scheme 1. Synthesis of (±) cis-bisamido epoxides, Reagents and conditions: a) TFAA, DCM, quantitative b) 4-butyl aniline, ether, followed by Ac2O, AcONa, 31–41% (over 2 steps) c) NH_3 (aq), MeOH, 67–75%.

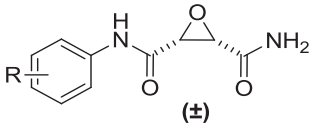
ring opened with hexamethyldisilizane to produce acid **9**. Amide coupling with 1-hydroxybenzatriaozle monohydrate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide methiodide was then performed with a number of amines (Scheme 2) to produce a small library of inhibitors **10**–**20**, which displayed good potency towards FXIII-A (Table 1).

The inhibition appeared to be irreversible as the activity of enzyme that had been exposed to the inhibitors could not be restored following exhaustive dialysis, consistent with irreversible formation of an enzyme-inhibitor covalent bond resulting from attack of C314 onto the epoxide moiety of the inhibitors. Additionally, the biological data revealed some telling insights into the nature of the bis-amido epoxide binding mode. In particular, inhibitor **10**, which contains a butyl chain, exhibits relatively weak inhibition. Although containing a side chain modelled, in terms of overall length, on cerulenin itself, butyl-based system **10** exhibits much weaker inhibition (with the exception of compound **14**), than compounds **11**–**17**. The weak inhibition produced by epoxide **10** can be interpreted as an entropic effect on binding the conformationally flexible butyl chain to the FXIII-A active site. The remainder of this series of inhibitors display potencies in broadly the same range (0.1–0.5 μM) with two notable exceptions. Firstly, unsubstituted aniline derivative **14** induced much weaker inhibition (0.72 μM) of FXIII-A. This indicates that substituents on the phenyl ring are playing a role in binding to FXIII-A. Secondly, the 3-phenoxy, and 3-chloro-4-bromo analogues **13** and **16** are at least ten-fold more potent (IC_{50} = 4 nM) than any other derivative assayed. The chloro-substituent at the 3-position of **16** is evidently important in producing a substantial increase in affinity, as emphasised by comparison to 4-bromo analogue **15** which has an IC_{50} of 130 nM. It is interesting to note that 3,4-dimethyl analogue **17** produced comparatively weak inhibition of FXIII-A, thus suggesting that the



Scheme 2. Alternative synthesis of (±) cis-bisamido epoxides, reagents and conditions: a) Hexamethldisilazine, THF, 55% b) RNH_2, EDC•MeI, HOBt•H_2O, 19–69%.

Table 1
In vitro activity data of bis-amido epoxide derivatives toward plasma Factor XIII-A.



(±)

Compound	R	IC_{50} (μM)	
		FXIII-A	TGII
10	4-Bu	0.22 ± 0.13	0.15 ± 0.092
11	4-OPh	0.055 ± 0.011	0.046 ± 0.070
12	3-Cl	0.048 ± 0.053	0.048 ± 0.054
13	3-OPh	0.0040 ± 0.053	0.050 ± 0.067
14	H	0.72 ± 0.13	1.1 ± 0.043
15	4-Br	0.13 ± 0.019	0.057 ± 0.031
16	3-Cl, 4-Br	0.0040 ± 0.00021	0.0096 ± 0.018
17	3,4 di-Me	0.11 ± 0.021	0.13 ± 0.025
18	4-$CH_2CH_2NHSO_2$(2-NO_2Ph)	0.090 ± 0.043	0.088 ± 0.024
19	4-(4-$NO_2C_6H_4O$)	0.43 ± 0.0097	N.D
20	4-(4-$NH_2C_6H_4O$)	0.086 ± 0.073	0.048 ± 0.029
21	4-(C_6H_4)-4-NHCO(4-F C_6H_4)	0.10 ± 0.016	0.15 ± 0.092

IC_{50} values were generated by non linear regression of percent inhibition vs log inhibitor concentration for at least four different concentrations.

halogen groups present in inhibitor **16** are more suitable substituents than alkyls for producing potent inhibitors of FXIII-A. In addition, the potent inhibition observed for 3-phenoxy derivative **13** suggests that larger groups at the 3-position of the arylamide moiety of these inhibitors are well tolerated within the FXIII-A active site. The corresponding acids of the carboxamide inhibitors, obtained from direct opening of anhydride **4** with the corresponding amines, were also assayed against FXIII-A and showed no activity at 100 μM (data not shown), underlining the importance of the primary amide group and reinforcing the modelling which predicts that the primary amide group is making H-bonding contacts within the active site (Fig. 3). Additionally, ion pair repulsion between the C314 thiolate and the carboxylate present in the acidic compounds could also contribute to this lack of potency.

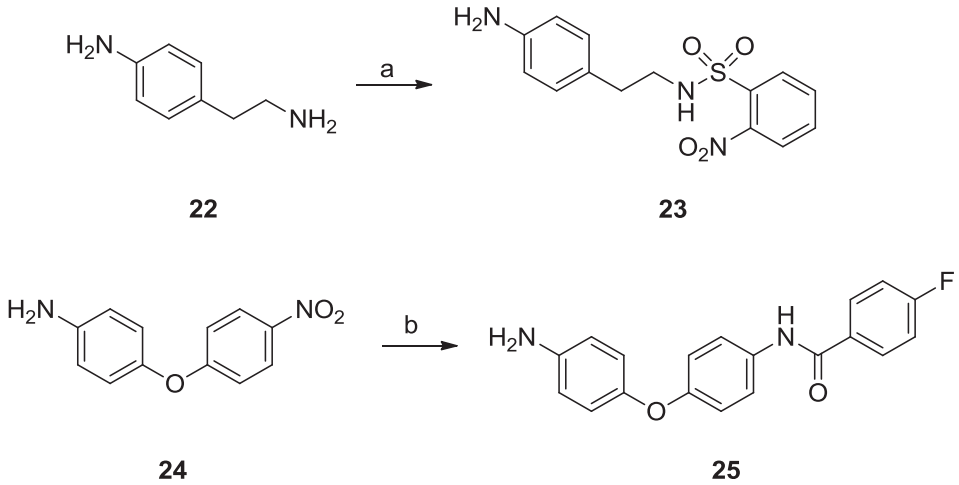
Modelling had also indicated that the rather elongated binding pocket of FXIII-A may be able to accommodate longer side-chains than those within molecules **10**–**17**, **19** and **20**. Therefore, sulfonamide derivative **18** was prepared, in which the side chain possesses a similar structure to that within the previously reported thiadiazole based inhibitor **1** [10]. The sulphonamide-containing amine precursor used for the synthesis of inhibitor **18**, was prepared from amine **22** as shown in Scheme 3. Additionally, analogue **21**, based around an extension of phenoxy derivative **11**, *via* the formation of an amide at the 4-position, was targeted. Specifically, amide **25** was synthesised as depicted in Scheme 3 and was then coupled to acid **9** using the standard conditions, to yield molecule **21**.

However, sulfonamide **18** showed similar potency to the simpler derivatives suggesting that either the compound does not effectively occupy the FXIII-A pocket or that the flexibility in this longer side chain imparts an entropic penalty on binding, thus lowering the overall affinity relative to the increase in size. Analogue **21** was also found to possess similar potency to the simpler analogues (Table 1). Perhaps in this case, a slightly more flexible system is required in order to allow the 'tail' portion of the inhibitors to effectively fill the binding pocket, although as indicated by sulphonamide **18**, highly flexible alkyl chains may show relatively modest inhibition.

Gratifyingly, as for cerulenin itself (Fig. 1), it was found that the FXIII-A inhibitory activity of all of the bisamido epoxides did not significantly change in the presence of GSH (See Supporting Information), underlining the stability of these systems in the presence of simple thiols. Further studies, (not shown), similarly indicated a low rate of spontaneous hydrolysis of the epoxide to the diol in neutral aqueous solution.

3. Selectivity

In order to assess the selectivity of the inhibitors, they were assayed against two other types of cysteine-based enzymes. Firstly, all compounds were tested against the evolutionarily distant enzyme cathepsin S, in order to probe the ability of the epoxides to inhibit other cysteine-protease inhibitors. Gratifyingly, all compounds showed negligible inhibition of cathepsin S at concentrations as high as 100 μM, which equates to around 10,000 fold selectivity for FXIII-A. Having established that the inhibitors show a level of selectivity for the active site of FXIII-A, we wished to determine the extent of this selectivity *via* comparison of the FXIII-A inhibitory activity with that shown with transglutaminase II (TGII), which is a transglutaminase with a remarkably close



Scheme 3. Synthesis of anilines required to prepare derivative **18** and **21**, reagents and conditions: a) 2-Nitrobenzenesulfonyl chloride, NEt_3, DCM, 44% b) 4-fluorobenzoyl chloride, pyridine followed by H_2 Pd/C, MeOH, 50% over 2 steps.

structural similarity to FXIII-A [17]. However, as shown in Table 1, the inhibitors also displayed similar levels of potency to TGII, in keeping with the very high structural similarities between the two enzymes. Further studies may achieve selectivity by targeting a binding cleft specific to TGII, but alternatively since FXIII-A is the only transglutaminase expressed at measurable levels in plasma, specificity might also be achieved by confining the inhibitor to this compartment.

4. Conclusions

Molecular modelling of the initial binding mode of a natural product-based FXIII-A inhibitor cerulenin, indicated that simple substitution of a ketone for an arylamide moiety could yield analogues which were predicted to be more potent but which would also be more synthetically accessible, and a number of these *cis*-bisamido epoxides were readily prepared using a simple synthetic procedure. Assaying of these compounds revealed that a significant increase in inhibitory potency could be achieved (up to $IC_{50} = 4$ nM) compared to that displayed by cerulenin (5 μM). A limited study of selectivity revealed that, whilst these inhibitors appear to be selective for FXIII-A compared to the unrelated cysteine protease cathepsin S, they could not differentiate between FXIII-A and TGII, which are closely related members of the transglutaminase family. As such differentiation is desirable in a clinical setting, more work is needed in order to optimise the structures of these inhibitors as potential antithrombotic drug leads.

Acknowledgement

The authors would like to thank Heart Research UK for a Studentship (to C.A.A).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.05.019.

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