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RATIONAL DESIGN OF BOROPEPTIDE THROMBIN INHIBITORS: β,β-DIALKYL-PHENETHYLGLYCINE P2 ANALOGS OF DUP 714 WITH GREATER SELECTIVITY OVER COMPLEMENT FACTOR I AND AN IMPROVED SAFETY PROFILE[1]

John M. Fevig,* Joseph Buriak, Jr., Joseph Cacciola, Richard S. Alexander, Charles A. Kettner, Robert M. Knabb, James R. Pruitt, Patricia C. Weber,‡ and Ruth R. Wexler

The DuPont Merck Pharmaceutical Company, P.O. Box 80500, Wilmington, DE 19880-0500

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Abstract: The potent boropeptide thrombin inhibitor DuP 714 caused side effects in laboratory animals that appear to be related to its ability to inhibit complement factor I, thereby activating the complement cascade. Using X-ray crystal structure information, we have designed compounds that have greater selectivity for thrombin over factor I and that have reduced tendency to produce these side effects.

The serine protease thrombin is a critical enzyme in the blood coagulation cascade and, consequently, inhibitors of thrombin have been pursued as potential antithrombotic agents.[2] Ac-*D*-Phe-Pro-boroArg-OH (DuP 714) (Figure 2) is a potent (K_i = 0.04 nM), orally active thrombin inhibitor that is effective against both arterial and venous thrombosis in animal models.[3] However, DuP 714 also causes hypotension and elevated levels of serum transaminases following bolus iv dosing. Further studies indicated that DuP 714 also caused transient thrombocytopenia and leukopenia, and that it caused localized inflammation in response to local injections.

Extensive *in vitro* and rat *in vivo* studies[4] aimed at determining the mechanism of these toxic side effects initially focused on mast cell degranulation and/or complement activation as potential inflammatory mediators. The histamine release characteristic of known mast cell degranulators such as compound 48/80[5] was not observed upon administration of DuP 714, suggesting that DuP 714 is not acting as a mast cell degranulator. However, depletion of complement with cobra venom factor (CVF)[6] prior to administration of DuP 714 blocked the hypotension, serum transaminase elevations and thrombocytopenia normally observed.[4] Additionally, DuP 714 was found to activate complement *in vitro* at 1-10 μM, indicated by increased serum levels of SC5b-9, anaphylatoxin C3a and factor Bb, a marker of activation of the alternate complement pathway. Examination of the complement cascade (Figure 1) reveals that, of its many serine proteases, factor I alone has an attenuating role, which involves the inactivation of C3b. Subsequently, DuP 714 was found to be a potent inhibitor of complement factor I (IC_{50} = 10 nM).[7] Therefore, it was concluded that inhibition of complement factor I by DuP 714 allows for rapid amplification of the alternate pathway of the complement cascade, which ultimately leads to the production of C3a, C5a and SC5b-9.[4] The transient high levels of anaphylatoxins C3a and C5a presumably either directly or indirectly cause the observed side effects. As a means of reducing these undesirable side effects, we sought to design inhibitors with greater selectivity for thrombin over factor I. This manuscript will describe our rational design efforts in this area that have indeed led to compounds that are more selective for thrombin over factor I and that have less propensity to cause hypotension and serum transaminase elevations.[8]

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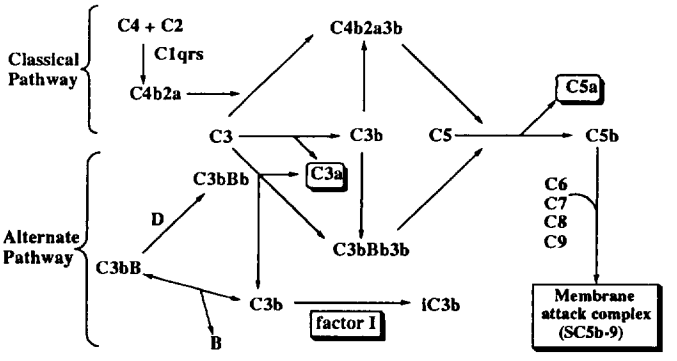
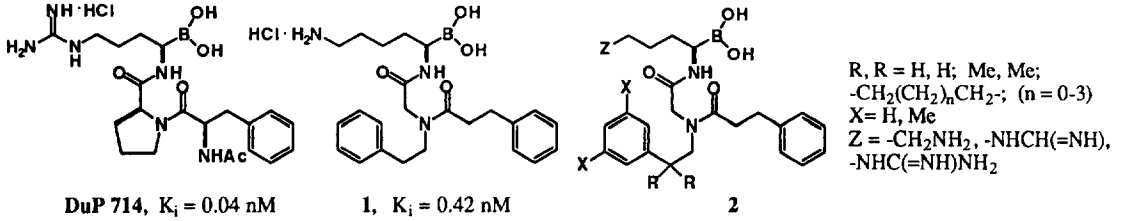
Figure 1. The Complement Cascade



Our design efforts were based upon a comparison of the amino acid sequences of thrombin and factor I,[9] which revealed that both enzymes contained similarly shaped and charged residues in most areas of the ligand binding region. However, the two enzymes differ at residue 99, the side chain of which projects toward the aryl binding pocket.[10] Thrombin has a leucine at this position while factor I has a longer tyrosine residue. The longer Tyr99 residue should make the aryl binding pocket of factor I smaller than that of thrombin, thereby allowing the opportunity for achieving greater selectivity by more completely filling the larger thrombin pocket. Factor Xa, another integral serine protease in the coagulation cascade, also contains the Tyr99 for Leu99 substitution while being similar to both thrombin and factor I in the other ligand binding pockets. Due to the apparent similarity between factor Xa and factor I, we elected to use the X-ray coordinates of factor Xa[11] as a model in our design of more selective thrombin inhibitors.

The boropeptide [*N*-hydrocinnamoyl-N-phenethyl]Gly-boroLys-OH (**1**)[12] (Figure 2) was ideally suited as a starting point for these rational design efforts. The X-ray crystal structure of the thrombin:**1** complex[12,13] reveals that the *N*-phenethyl residue of **1** occupies a position adjacent to residue Leu99, with the phenyl ring being engaged in a favorable aromatic edge-to-face interaction[14] with the indole side chain of Trp215 in the aryl binding pocket. We expected that the *N*-phenethyl residue would therefore serve as a handle on which to append additional functionality to interact with this region of the binding pocket. An overlay of the coordinates of the thrombin:**1** complex with those of factor Xa (Figure 3a) reveals a distance of 4.8 Å between the β carbon of the *N*-phenethyl residue of **1** and a Leu99 terminal methyl group. The Tyr99 side chain of factor Xa projects toward the *N*-phenethyl residue so that the distance between the β carbon and the Tyr99 phenolic oxygen in this model is 2.5 Å. Working on the assumption that the overall conformation of factor I will be similar to that of factor Xa, we expected that disubstitution of the *N*-phenethyl β carbon of **1** would result in compounds which would suffer from steric interactions with Tyr99 upon binding to factor I. Thrombin, with the smaller Leu99

Figure 2

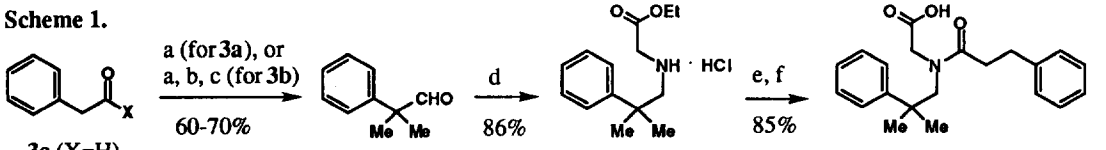


DuP 714, K_i = 0.04 nM **1**, K_i = 0.42 nM **2**

residue, was expected to be more tolerant of substitution at this position. As shown in Figure 2, we chose initially to make compounds **2** in which the β carbon was disubstituted with hydrophobic groups to avoid both the introduction of a new chiral center and any potential hydrogen bonding interactions with Tyr99.

The β,β-disubstituted phenethylglycines required for preparation of compounds **2** were prepared as illustrated for *N*-hydrocinnamoyl-*N*-[(2,2-dimethyl-2-phenyl)ethyl]glycine **6** shown in Scheme 1. The aldehyde **3a** could be directly dialkylated with 2.2 equiv of methyl iodide and 2.2 equiv of KO*t*-Bu to give **4**[15] in about 65% yield. Alternatively, dialkylation of ester **3b** followed by LAH reduction and PCC oxidation gave aldehyde **4** in comparable overall yield. The required cycloalkyl aldehydes related to **4** were prepared from commercially available 1-phenyl-1-cycloalkanecarboxylic acids by an analogous two step adjustment of oxidation state. Reductive amination of **4** with glycine ethyl ester hydrochloride salt and sodium cyanoborohydride smoothly afforded the amine salt **5**. Acylation with hydrocinnamoyl chloride followed by saponification of the ester gave the desired glycine derivative **6**. The conversion of **6** and related derivatives to the final boropeptide inhibitors followed established procedures and was straightforward.[3a,12,16]

Scheme 1.



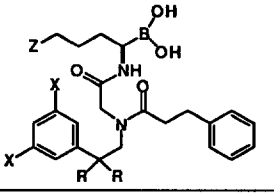
Reagents: (a) MeI (2.2 equiv), KO*t*-Bu (2.2 equiv), THF; (b) $LiAlH_4$, Et_2O, 0°C; (c) PCC, CH_2Cl_2; (d) GlyOEt · HCl, $NaCNBH_3$, MeOH; (e) $PhCH_2CH_2COCl$, NMM, THF; (f) KOH, $MeOH/H_2O$, reflux.

In Table 1 is shown binding and selectivity data for analogs prepared according to our design. Liver enzyme elevation (ALT) data is included as a measure of toxicity. In general, β,β-disubstitution of the phenethyl residue led to compounds which had greater selectivity for thrombin over factor I, as indicated by increases in the calculated selectivity ratio (factor I IC_{50}/thrombin K_i). The same basic trend is seen in the selectivity for thrombin over factor Xa, which lends support to our use of factor Xa as a model of factor I. Some exceptions to this trend are observed in the borolysine series **1**, **7**-**9**. The β,β-dimethyl analog **7** is equipotent to **1** toward thrombin and factor I, so there is no increase in the selectivity ratio. However, **7** is more selective versus factor Xa than is **1**. Also, the cyclopropyl analog **8** is more selective for thrombin over both factor I and factor Xa than is the bulkier cyclopentyl analog **9**, although the loss of factor I and factor Xa affinity for **9** relative to **1** is still consistent with our model and with our design. The effect of β,β-disubstitution is best observed within the (formamidino)boroornithine[12] series **10**-**13**. The β,β-dimethyl and cyclopropyl analogs **11** and **12**, respectively, are nearly equipotent to the unsubstituted analog **10** toward thrombin but have lower affinities for factor I and factor Xa. The bulkier cyclopentyl analog **13** begins to lose affinity for thrombin while dramatically losing affinity for factor I. Thus, the selectivity ratios for **13** increase > fivefold against factor I and about eightfold against factor Xa relative to the unsubstituted analog **10**. The cyclopropyl analogs are consistently more selective for thrombin over factor I than are the corresponding β,β-dimethyl analogs.

The 3,5-dimethylphenethyl series **16-21** also follows the same selectivity trend. The β,β-dimethyl analogs **17**, **19**, and **21** are nearly equipotent toward thrombin relative to their unsubstituted counterparts **16**, **18**, and **20**, respectively, but are considerably less potent toward factor I and factor Xa, with increases in the selectivity ratio versus factor I ranging from fourfold to > fifteenfold and increases in the selectivity ratio versus factor Xa ranging from eightfold to tenfold. Indeed, the effect of β,β-dimethyl substitution in this series is even greater than in the phenyl series, where β,β-dimethyl substitution resulted in two- to threefold increases in selectivity ratios. This effect might result from one of the *meta* methyl groups being positioned over Trp215, which may sterically force the phenethyl tether closer to Tyr99 and enhance the effect of β,β-disubstitution.[17]

The measured levels of alanine transaminase (ALT) reported in Table 1 are also worthy of note. In accord with the relationship between factor I inhibition and toxicity, only the most potent factor I inhibitors, namely DuP 714 and **14**, show significant elevations in ALT levels. The borolysines **1** and **7-9** and the (formamidino)boroornithines **10-13** are free from ALT elevations, which, we believe, reflects the fact that they are weak factor I inhibitors relative to DuP 714 and **14**. Interestingly, even the unsubstituted phenethyl analogs **1** and **10** are relatively weak factor I inhibitors, possibly because even the unsubstituted phenethyl residue provides some degree of steric interaction with the Tyr99 of factor I (Figure 3a).

Table 1. Binding data, selectivity data and toxicity data for β,β-disubstituted phenethylglycine derivatives.



				Binding Data			Selectivity Ratios		Toxicity Data
Cmpd#	X	Z	R, R	Thrombin K_i (nM) [a]	factor I IC_{50} (nM) [b]	factor Xa K_i (nM) [c]	fI IC_{50}/ thr K_i	fXa K_i/ thr K_i	ALT Levels [d] (20μmol/kg)
DuP 714	-	$-NHC(=NH)NH_2$	-	0.04	10	9	250	225	287(2μmol/kg)
1	H	$-CH_2NH_2$	H, H	0.42	1500	130	3570	310	60
7	H	$-CH_2NH_2$	Me, Me	0.36	1300	320	3611	889	53
8	H	$-CH_2NH_2$	$-(CH_2)_2-$	0.43	4300	438	10,000	1019	60
9	H	$-CH_2NH_2$	$-(CH_2)_4-$	2.4	19,000	1390	7916	579	66
10	H	-NHCH(=NH)	H, H	0.89	2730	22	3067	25	55
11	H	-NHCH(=NH)	Me, Me	1.2	6900	80	5750	67	70
12	H	-NHCH(=NH)	$-(CH_2)_2-$	0.83	7700	300	9277	361	66
13	H	-NHCH(=NH)	$-(CH_2)_4-$	3.0	>50,000	630	>16,667	210	60
14	H	$-NHC(=NH)NH_2$	Me, Me	0.06	130	99	2166	1650	211
15	H	$-NHC(=NH)NH_2$	$-(CH_2)_2-$	0.06	540	46	9000	767	89
16	Me	$-CH_2NH_2$	H, H	0.28	170	54	607	193	nt
17	Me	$-CH_2NH_2$	Me, Me	0.41	2450	810	5975	1976	78
18	Me	-NHCH(=NH)	H, H	0.37	400	30	1080	81	nt
19	Me	-NHCH(=NH)	Me, Me	0.53	2390	392	4509	740	70
20	Me	$-NHC(=NH)NH_2$	H, H	0.58	<40	15	<69	26	nt
21	Me	$-NHC(=NH)NH_2$	Me, Me	0.30	320	62	1066	207	nt

a) Values for inhibitory constant (K_i) were determined as described in ref 3a. The majority of compounds are slow-binding inhibitors. Reported values are the averages of at least duplicate measurements after steady state velocities were reached.
b) Values for IC_{50} were determined as described in ref 7 and are averages of at least duplicate measurements.
c) Values for inhibitory constant (K_i) were determined as described in ref 3a and are averages of at least duplicate measurements.
d) ALT = alanine transaminase. Control level is 57. nt = not tested.

Figure 3a. **Figure 3b.**

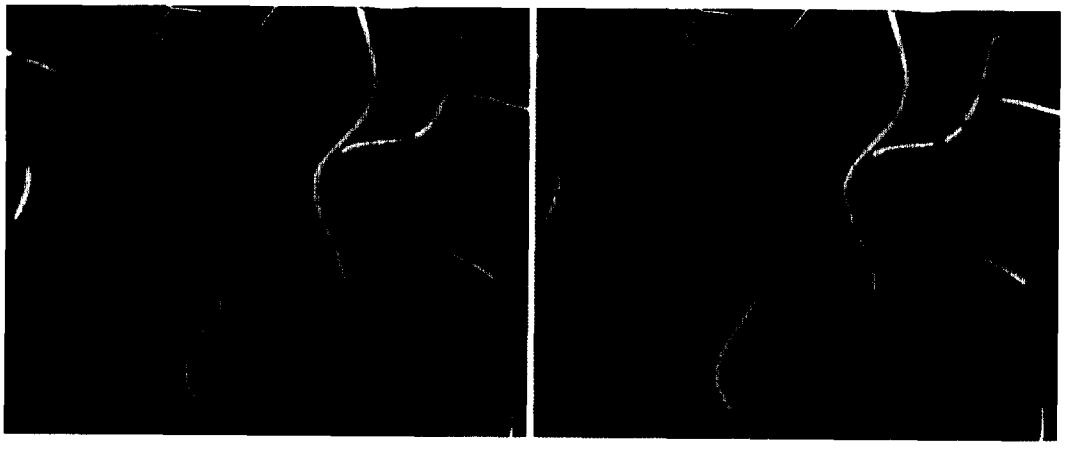


Figure 3a. Factor Xa coordinates (red) superimposed onto coordinates of **1** (green) bound to thrombin (yellow). The distance from the phenethyl β-carbon of **1** to the methyl group of Leu99 of thrombin is 4.8 angstroms (pink dashed line) and to the phenolic oxygen of Tyr99 of factor Xa is 2.5 angstroms (yellow dashed line). **Figure 3b.** Factor Xa coordinates (red) superimposed onto coordinates of **1** (green) and **7** (blue) bound to thrombin (yellow). The distance from the methyl group of **7** to the phenolic oxygen of Tyr99 of factor Xa in this model is 1.4 angstroms.

The additional effect of factor I selectivity on hypotension is illustrated in Table 2 for a series of boroarginine inhibitors. The very potent factor I inhibitor DuP 714 causes dramatic ALT elevations along with hypotension after an iv bolus dose of 2 μmol/kg in rats. The boroarginine **14**, which is thirteenfold less potent toward factor I, causes dramatic ALT elevations along with hypotension only after a tenfold higher dose. The boroarginine **15**, with the same thrombin affinity as **14** but with fourfold lower factor I affinity, causes only slight ALT elevations and no hypotension at the 20 μmol/kg dose. Thus, these results effectively demonstrate that, among structurally-related compounds having comparable thrombin affinity, the toxic side effects of the boropeptides can be attenuated *in vivo* by increasing the selectivity over complement factor I.

Table 2.

Compound	Thrombin K_i (nM)	Factor I IC_{50} (nM)	ALT Levels rat i.v. bolus (dose)	Hypotension Data* rat i.v. bolus (dose)
DuP 714	0.04	10	287 (2 μmol/kg)	Yes (2 μmol/kg)
14	0.06	130	211 (20 μmol/kg)	Yes (20 μmol/kg)
15	0.06	540	89 (20 μmol/kg)	No (20 μmol/kg)

* Yes defined as a > 40 mmHg drop in blood pressure.

To determine whether the disubstituted analogs listed in Table 1 are binding in the manner in which they were designed, we solved the X-ray crystal structure of **7** bound to thrombin. Although this compound has about the same factor I potency as the corresponding unsubstituted analog **1**, it is threefold more selective versus factor Xa than is **1**. Figure 3b shows an overlap of the coordinates of the thrombin:**7** complex[13] with those of both the thrombin:**1** complex[13] and factor Xa. While the overall binding conformations of **7** and **1** are similar,

the phenethyl residue of **7** has moved slightly, presumably to accommodate the additional methyl groups. Also, the orientation of the phenyl residue with respect to Trp215 has changed, but the edge-to-face interaction is still maintained at approximately the same inter-ring angle. The measured distance of 1.4 Å between the phenethyl methyl group of **7** and the oxygen of Tyr99 of factor Xa in this model lends support to our hypothesis and to our results regarding the increased selectivity of substituted analogs of **1**. Presumably, binding of the dialkylated phenethyl analogs to factor I or factor Xa in a conformation similar to **1** would be disrupted by steric interactions between the added alkyl groups and Tyr99. To accommodate these added groups the phenethyl residue might bind in a different manner, which might be expected to disrupt the favorable edge-to-face interaction with Trp215.

In summary, we have used X-ray crystal structure information to design substituted analogs of the boropeptide **1** which have greater selectivity for thrombin over complement factor I. The resulting inhibitors have less tendency to cause the side effects which we believe are mediated by inhibition of complement factor I. This work is an example of how rational drug design can be used to target a specific binding interaction, based on a single amino acid substitution, which can dramatically alter the selectivity and biological activity profile of a promising series of enzyme inhibitors.

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‡ Current address: Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033.
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