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Structure-Based Design, Synthesis and Evaluation of Novel Peptidic Inhibitors of Thrombin-Induced Activation of Platelets Aggregation

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Introduction

Thrombosis-related disorders such as myocardial infarction, stroke and pulmonary embolism remain a major cause of mortality and morbidity worldwide [1]. This has driven the interest on thrombin inhibitors as potential antithrombotic drugs [2]. However, to date, discovery of safe, selective and orally available inhibitors has proven difficult to accomplish, therefore limiting their therapeutic use [3,4]. Newer direct thrombin inhibitors (DTIs) are attempting to fine tune thrombin's activity by targeting allosteric sites or by site-specific targeting of clotting. Advancements in formulations and production processes have attempted to make traditional DTIs more cost effective to produce. Today, the research focused on optimizing anti-thrombosis drugs reveals a trend to develop a thrombin 'modulator' rather than an 'inhibitor [2-4]. Recently, our research group described the biochemical and structural characterization of three peptidic non-covalent direct thrombin inhibitors (DTI) that contain the common sequence D-Phe(P_3)-Pro-(P_2)-D-Arg(P_1)-P_1'-$CONH_2$ ([1] and Figure 1). The three-dimensional structures of three complexes of human alpha-thrombin with the lead peptidic competitive inhibitors (with L-isoleucine, L-cysteine or D-threonine at the P1' position) highlighted all inhibitors adopting a substrate-like orientation in the active site of thrombin [1]. Moreover, other collaborators in the field developed biomaterials with enhanced haemocompatibility containing our lead peptidic DTI [D-Phe-Pro-D-Arg-D-Thr-$CONH_2$] (fPrt) [6]. The research proved that the immobilization of the thrombin inhibitor (fPrt) onto nanostructured surfaces induces selective and reversible adsorption of albumin, delaying the clotting time when compared to peptide-free surfaces [5]. To further improve the potency and selectivity of the peptidic DTI and assess their future potential as scaffolds for developing biomaterials with improved haemocompatibility for the blood-contacting medical devices, we performed a structure-based drug design (SBDD) and structure-activity relationship (SAR) evaluation of novel peptidic DTI by optimizing the P3 position within the original scaffold "D-Phe(P3)-Pro-(P2)-D-Arg(P1)-P1'-$CONH_2$" with other un-natural D-Phe analogs (such as D-3,3-Diphenylalanine) (Figure 2).

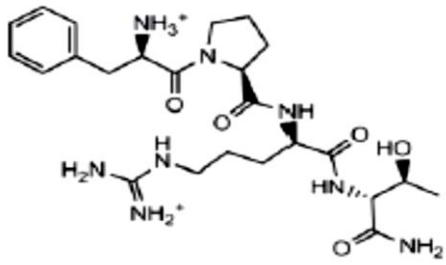

Fig. 1. Structure of the lead DTI [D-Phe-Pro-D-Arg-D-Thr-$CONH_2$](fPrt) characterized in complex with human alpha thrombin (3U8O.pdb [1]).

Results and Discussion

Thrombin is known to induce the activation of platelets to aggregate by binding to and cleaving the extracellular *N*-terminal domains of protease-activated receptors 1 and 4 (PAR1 and PAR4) [3]. To date many DTI proved to also be potent inhibitors of the thrombin mediated activation of platelet aggregation. Such DTI can be used as pharmacological agents for the management of acute coronary syndrome (ACS) [3].

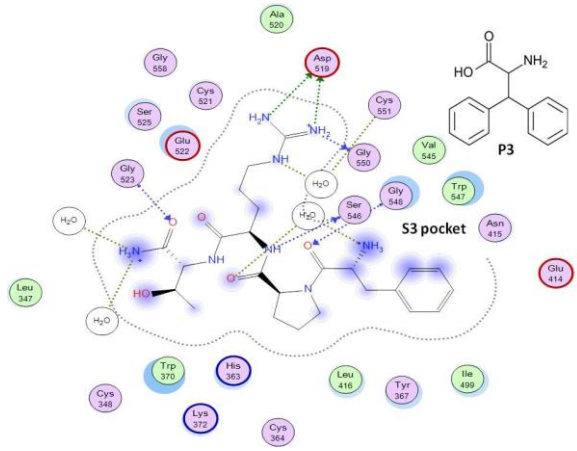

Fig. 2. Illustration of the lead compound [D-Phe(P3)-Pro(P2)-D-Arg(P1)-D-Thr(P1')-CONH$_2$] DTI (fPrt) in complex with human alpha thrombin (3U8O.pdb) (1). The main interactions with the amino acids within 5Å of the thrombin's active site are displayed. The S3 pocket is occupied by D-Phe which is replaced by D-3,3-Diphenylalanine (DIP)in P3 position.

This research presents the structure-based drug design (SBDD), synthesis and evaluation of novel tetrapeptides DTI inhibitors of thrombin-activated platelet aggregation. Analogs of the lead DTI, [D-Phe(P_3)-Pro(P_2)-D-Arg(P_1)-D-Thr(P_1')-CONH$_2$], have been designed to improve the hydrophobic driven interactions with the S3 pocket of thrombin by replacing the D-Phe (in P3) with D-3,3-Diphenylalanine (DIP) among other unnatural amino acids (such as D-3,5-difluorophenylalanine, (L)/(D)-Tic [1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid], (L)/(D)-Thi [Thienylalanine], D-Naphthylalanine (D-Nal(1) and D-Nal(2)) and 1,2,3,4-tetrahydronorharman-3-carboxylic acid (D-Tpi). The new peptidic DTIs were designed and developed by means of "*in silico*" SBDD approach where the X-ray structure of the DTI lead compound [D-Phe-Pro-D-Arg-D-Thr-CONH$_2$] (fPrt) in complex with human alpha-thrombin (3U8O.pdb) was used as a template for rigid docking experiments performed with AutoDock Vina [6]. The docking experiments provided the free energy of interaction between each ligand and thrombin template (Table 1).

The new lead DTIs were synthesized using standard solid-phase fluorenylmethyloxycarbonyl (Fmoc) chemistry in collaboration with Karebay Biochem, Inc. as described earlier (1). Two types of binding experiments were performed to assess the inhibitory constant (Ki): (1) kinetics of alpha-thrombin inhibition of chromogenic substrate S2238; and (2) surface plasmon resonance (SPR) with immobilized alpha-thrombin. All D-3,3-Diphenylalanine-DTI analogs competitively inhibited alpha-thrombin's cleavage of the S2238 chromogenic substrate with K(i) of 500-24 nM (Table 1). Moreover, the kinetic constants and the binding affinities for the interaction between the two lead synthetic peptides and immobilized thrombin monitored by surface plasmon resonance (SPR) revealed that the Kd (dissociation constant) was in the order of 290 to 40-50 nanomolar (Table 2). Remarkably the new DTIs proved to inhibit the alpha thrombin activation of platelets aggregation.

Table 1. Summary of some of the lead peptidic DTIs and the corresponding experimental and predicted Ki (nM).

Peptide sequence (ID)	*Experimental Ki (nM)*	*Predicted (AutoDock Vina) Ki (nM)*
D-3,3-Diphenylalanine-Pro-D-Arg-D-Cys-CONH$_2$	65.5 $\pm$ 0.3	40.2
D-3,3-Diphenylalanine-Pro-D-Arg-D-Ala-CONH$_2$	130.6 $\pm$ 0.5	85
D-3,3-Diphenylalanine-Pro-D-Arg-D-Thr-CONH$_2$	104.4 $\pm$ 1.5	50.2
D-3,3-Diphenylalanine-Pro-D-Arg-D-Val-CONH$_2$	102.2 $\pm$ 5.5	45
D-3,3-Diphenylalanine-Pro-D-Arg-D-Ile-CONH$_2$	64.2 $\pm$ 2.5	35
D-3,3-Diphenylalanine-Pro-D-Arg-D-Leu-CONH$_2$	540.2 $\pm$ 2.4	350
D-3,3-Diphenylalanine-Pro-D-Arg-D-Thi-CONH$_2$	312.5 $\pm$ 0.8	120

Table 2. Summary of kinetic constants and binding affinities for the interaction between two synthetic DTI peptides and immobilized thrombin monitored by surface plasmon resonance (SPR). K_D was calculated as the ratio (k_d/k_a) of dissociation and association rate constants as $K_A = K_D^{-1}$. Sensorgrams were analyzed with BIA evaluation 4.1.1 software and the constants were determined by global fitting of the data using the Langmuir binding model ($k_d/k_a = K_A = K_D^{-1}$).

Peptide DTI	k_a ($M^{-1} \times s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (M)=Ki
D-3,3-Diphenylalanine-Pro-D-Arg-D-Cys-$CONH_2$	7.16×10^4	3.00×10^{-5}	2.39×10^9	4.19×10^{-10}
D-3,3-Diphenylalanine-Pro-D-Arg-D-Ala-$CONH_2$	1.82×10^5	5.36×10^{-2}	3.39×10^6	2.95×10^{-7}

Washed human platelets ($4x10^8$/ml from healthy donors) were incubated with Tyrode's buffer (vehicle), or each DTI peptide (50-0.1 uM final concentration) for 1 minute stirring at 37^0C followed by the stimulation with alpha-thrombin in presence of fibrinogen. The new DTI lead compounds (Tables 1 and 2) completely inhibited threshold alpha-thrombin-induced platelet aggregation at concentrations of 8000-25 nM (Figure 3). SAR analysis proved that selected amino acids substitutions in P1'position determine the potency of peptidic DTIs.

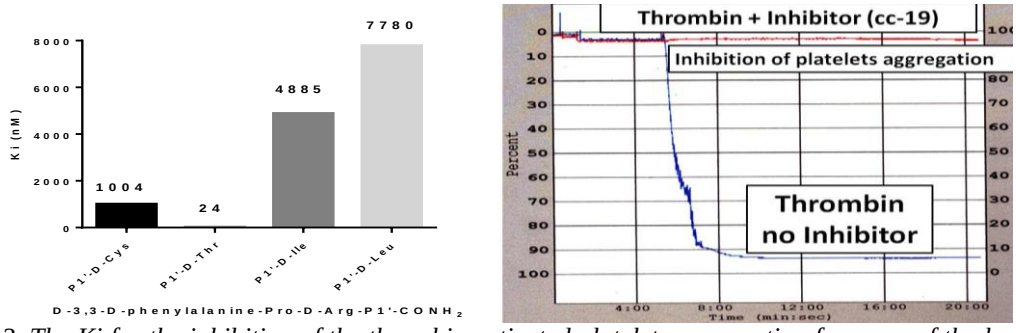

Fig. 3. The Ki for the inhibition of the thrombin activated platelets aggregation for some of the lead compounds containing DIP in P3 position showed that selected amino acids substitutions in P1' affect the potency of the peptidic DTIs (left). Representative platelets aggregation assay view in the absence and presence of one selected DTI (cc19: [D-3,3-Diphenylalanine-Pro-D-Arg-D-Thr-$CONH_2$]) (right).

This research demonstrated the proof of principle for using potent peptidic DTI (Ki in the hundreds to two digits nM range) to inhibit thrombin activated platelet aggregation. Specific amino acid substitutions required for activity against platelet aggregation have been identified for P1' positions, and lead compounds having D-3,3-Diphenylalanine in P3 position have been developed. These lead compounds completely inhibited threshold alpha-thrombin-induced platelet aggregation at concentration 10-24nM. These novel DTI tetrapeptides could be used as future pharamacophore scaffolds for the development of inhibitors of thrombin-mediated platelet aggregation for treatment of acute coronary syndrome (ACS).

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