Amino Acids (2010) 38:985–990
DOI 10.1007/s00726-009-0306-z

ORIGINAL ARTICLE

Design, synthesis and biological evaluation of non-peptide PAR_1 thrombin receptor antagonists based on small bifunctional templates: arginine and phenylalanine side chain groups are keys for receptor activity

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Received: 20 February 2009 / Accepted: 9 May 2009 / Published online: 31 May 2009

Abstract In the present study, we report the synthesis and biological evaluation of a series of new non-peptide PAR_1 mimetic receptor antagonists, based on conformational analysis of the $S_{42}FLLR_{46}$ tethered ligand (TL) sequence of PAR_1. These compounds incorporate the key pharmacophore groups in the TL sequence, guanidyl, amino and phenyl, which are essential for triggering receptor activity. Compounds **5** and **15** (50–100 μM) inhibited both TFLLR-amide (10 μM) and thrombin-mediated (0.5 and 1 U/ml; 5 and 10 μM) calcium signaling in a cultured human HEK cell assay.

Keywords Thrombin · PAR_1 receptor · SFLLR · Pharmacophore · Mimetics

Introduction

Receptors that mediate thrombin action are attractive drug discovery targets because of their involvement in cardiovascular pathophysiology (dysregulation of platelet aggregation and endothelial cell function) (Ogletree et al. 1994; Andrade-Gordon et al. 1999). The cellular actions of thrombin are, in large part, caused by the activation of proteinase-activated receptors (PARs) 1, 3 and 4 (Hollenberg and Compton 2002; Ramachandran and Hollenberg 2008; Nystedt et al. 1995; Kahn et al. 1998). The serine proteinase, thrombin, cleaves and activates cellular PAR_1 in many pathophysiological settings associated with hemostasis, tissue injury, tumor invasion and the proliferation of vascular smooth muscle and tumor cells. Thrombin cleaves the extracellular N-terminal peptide domain of human PAR_1 between Arg-41 and Ser-42 to expose a truncated N terminus bearing the peptide activation motif SFLLR (Matsoukas et al. 1996) that acts as a tethered receptor-activating ligand (Hollenberg and Compton 2002; Ramachandran and Hollenberg 2008). Synthetic peptides containing this amino acid sequence have full PAR_1 agonist properties independent of thrombin activation. The limited stability of peptides due to their peptidic nature often restricts their medical application.

PAR_1 peptide and non-peptide inhibitors have been reported in the last decade, some of which are already in clinical trials (Seiler and Bernatowicz 2003; Maryanoff et al. 2003; Chackalamannil et al. 2003; Selnick et al. 2003). Therefore, in the present study, we have attempted to synthesize a series of alternative new non-peptide PAR_1 mimetics (**5**, **12**, **15**, **18**), based on conformational analysis of the SFLLR tethered ligand sequence of PAR_1 using a series of templates aimed at inhibiting the cellular actions of thrombin (Alexopoulos et al. 2004). Structure–activity relationships (SAR) and amino acid substitutions in combination with NMR studies have determined the specific role of each amino acid in the native SFLLR sequence (Natarajan et al. 1995).

These synthetic compounds, which incorporate the essential tethered ligand pharmacophore groups (Alexopoulos et al. 1999) guanidyl, amino and phenyl a substituents built onto four different small bifunctional templates (piperidine, azetidine, cyclohexane and indole) (Zhang et al. 2003), were subsequently tested for biological

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activity in a PAR_1-dependent calcium signaling assay (Kawabata et al. 1999). Both compounds **5** and **15** (50–200 μM) were able to block thrombin and TFLLR-NH_2-triggered elevations in intracellular calcium via PAR_1. These active peptide mimetic antagonists show promise for the development of PAR_1-targeted agents as alternatives to those currently being considered for use in cardiovascular and cancer pathologies.

Materials and methods

General remarks

All of the solvents and reagents were obtained commercially and used as such, unless noted otherwise. TLC was performed on silica gel 60 F_{254} plates (Merck, Germany). HPLC was performed with Waters (Milford, MA, USA) system 600 controller equipped with Millenium 2.1 operating system using a Waters 996 diode array UV/vis detector. Purification of final products was performed with Waters Preparative HPLC (Waters Prep LC Controller) using SunFire Prep C18 column (50 × 100 mm) with 5 μm packing material. Separation was performed with stepped linear gradient from 5 to 60% AcN over 45 min at a flow rate of 12 mL/min. ^{1}H NMR spectra were recorded using Brucker 400 MHz spectometer. All chemical shifts (δ) were recorded as ppm and all samples were dissolved in DMSO-d_6. The coupling constants (J) were expressed in Hz. ESI-MS was performed with an electrospray platform (Waters) equipped with a Masslynx NT 2.3 operational system. Synthetic PAR_1-activating peptide, TFLLR-NH_2 (>95% pure by HPLC and mass spectral criteria) was obtained from the University of Calgary Peptide Synthesis Facility (peplab@ucalgary.ca). Human plasma thrombin (approx. 3,200 U/mg) was from Calbiochem (San Diego, CA, USA).

General method for the synthesis of compound 5

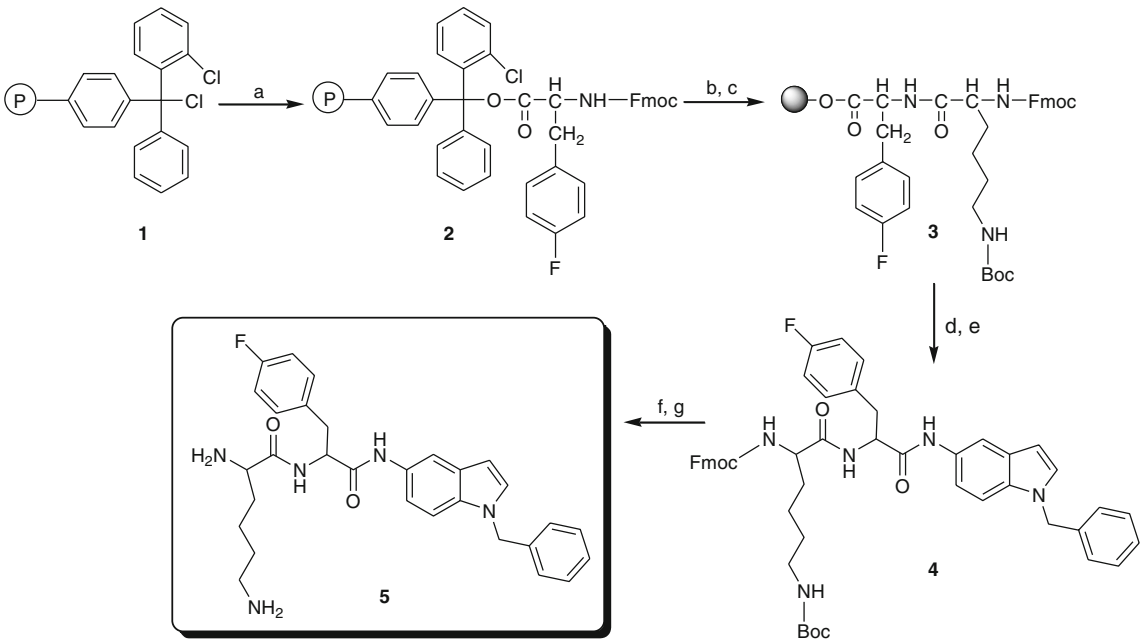
2-Chlorotritylchloride resin (1 g, 1.6 mmol Cl^-/g of resin) was left to swell in dry DCM (8 mL) for 30 min, *N*-Fmoc-4-fluoro-Phe-OH (0.338 g, 1 mmol), DIPEA (2.5 mmol) were added and stirred at RT. After 1 h, the suspension was filtered, washed successively with DCM/MeOH/DIPEA (17:2:1, v/v) (2 × 10 mL × 10 min), DMF (3 × 10 mL), *i*-Pro (2 × 10 mL), *n*-hexane (2 × 10 mL) and dried in vacuo for 24 h at RT. The loading of the amino acid/g of substituted resin was 0.7 mmol/g, calculated by weight and amino acid analysis. Removal of Fmoc group was achieved by the repetitive treatment with piperidine/DMF (1:5, 10 mL) for 10 and 20 min and the resin was thoroughly washed with DMF (3 × 10 mL), *i*-Pro (2 × 10 mL), *n*-hexane (2 × 10 mL). A solution of Fmoc-Lys(Boc)-OH (0.655 g, 1.4 mmol), HOBt (0.141 g, 1.05 mmol)/DIC (0.12 mL, 0.77 mmol) in DMF was added to the pre-swollen resin and the mixture was stirred at RT. After 3 h, the resin was filtered and washed as previously described. Cleavage from the resin using HFIP/DCM (3:7, 12 mL) for 2 h at RT, filtration, concentration of the solvent and precipitation by DEE, gave fully protected dipeptide as a white solid. The resulting solid (0.65 mmol) was dissolved in dry DCM, preactivated with HOBt (0.14 g, 1.04 mmol)/DCC (0.145 g, 0.715 mmol) for 30 min at 0°C and a solution of *N*-benzyl-5-amino indole (0.414 g, 0.65 mmol), DIPEA (0.51 mL) in DCM was added. After 7.5 h at RT the suspension was filtered off, the filtrate was diluted in DCM, washed successively with $NaHCO_3$ (1 × 5% w/v), citric acid (3 × 10% w/v), H_2O (×3), dried over Na_2SO_4 and concentrated in vacuo. Precipitation by DEE afforded crude **4** as a violet solid. Deprotection of Boc, Fmoc groups occurred with TFA/DCM (3:7) in the presence of scavengers TMSBr/anisole/TES for 1.5 h and piperidine/DCM (1:4) for 2 h. The solvents were removed and the crude **5** was isolated by trituration with DEE, purified by preparative RP-HPLC and lyophilized to furnish final product **5** as a pure violet solid (0.25 g, 0.49 mmol, overall 70%). A similar methodology was applied for the synthesized compounds **11**, **14**, **17**. All products were characterized by ^{1}H NMR and ESI-MS.

Data for compound **5**: ^{1}H NMR (400 MHz; DMSO-d_6) δ_{ppm} = 7.48–7.12 (m, 13H, Ar), 6.58 (d, 1H, J = 3.2 Hz), 5.37 (s, 2H), 4.96 (m, 2H), 3.57 (m, 1H), 3.23–3.12 (m, 2H), 2.67 (m, 2H), 1.78 (m, 2H), 1.46 (m, 2H), 1.29 (m, 2H); ESI-MS, *m/z*, 516.50 (M + 1), 518.45 (M + 3), 519.46 (M + 4).

General method for the guanylation of primary amine derivatives 11, 14, 17

TFA salt **14** (0.65 mmol), 1*H*-pyrazole-1-carboxamide hydrochloride (0.15 g, 1.04 mmol) and DIPEA (0.44 mL, 2.6 mmol) were dissolved in DMF sufficient to produce a final concentration approximately 2 M. The reaction mixture was stirred under nitrogen at RT while being monitored by TLC R_f = 0.35 in *n*-BuOH/AcOH/H_2O (4:1:1, v/v). After 12 h DEE was added to precipitate the crude **15**, which was collected, washed with DEE and dried. Purification by preparative RP-HPLC afforded pure **15** (0.53 mmol, 81%). Compounds **12**, **18** were prepared by a similar procedure.

Data for compound **15**: ^{1}H NMR (400 MHz; DMSO-d_6) δ_{ppm} = 8.32, 8.10 and 7.92 (3 br s, 4H), 7.73–7.30 (m, 4H), 4.25–4.11 (m, 3H), 3.06 (q, 2H, J = 6.8 Hz), 2.41–2.20 (m, 2H), 1.99–1.29 (m, 14H); ESI-MS, *m/z*, 450.07 (M^+), 451.39 (M + 1), 452.46 (M + 2).



Scheme 1 Reagents and conditions: **a** *N*-Fmoc-4-fluoro-Phe-OH, DIPEA, DCM, 1 h; **b** piperidine/DMF (1:5), 30 min; **c** Fmoc-Lys(Boc)-OH, HOBt/DIC, DMF, 3 h; **d** HFIP/DCM (3:7), 2 h; **e** *N*-benzyl-5-amino indole, HOBt/DCC, DIPEA, DCM, 0–25°C, 8 h; **f** TFA/DCM (3:7), TMSBr/anisole/TES, 1.5 h; **g** piperidine/DCM (1:4), 2 h

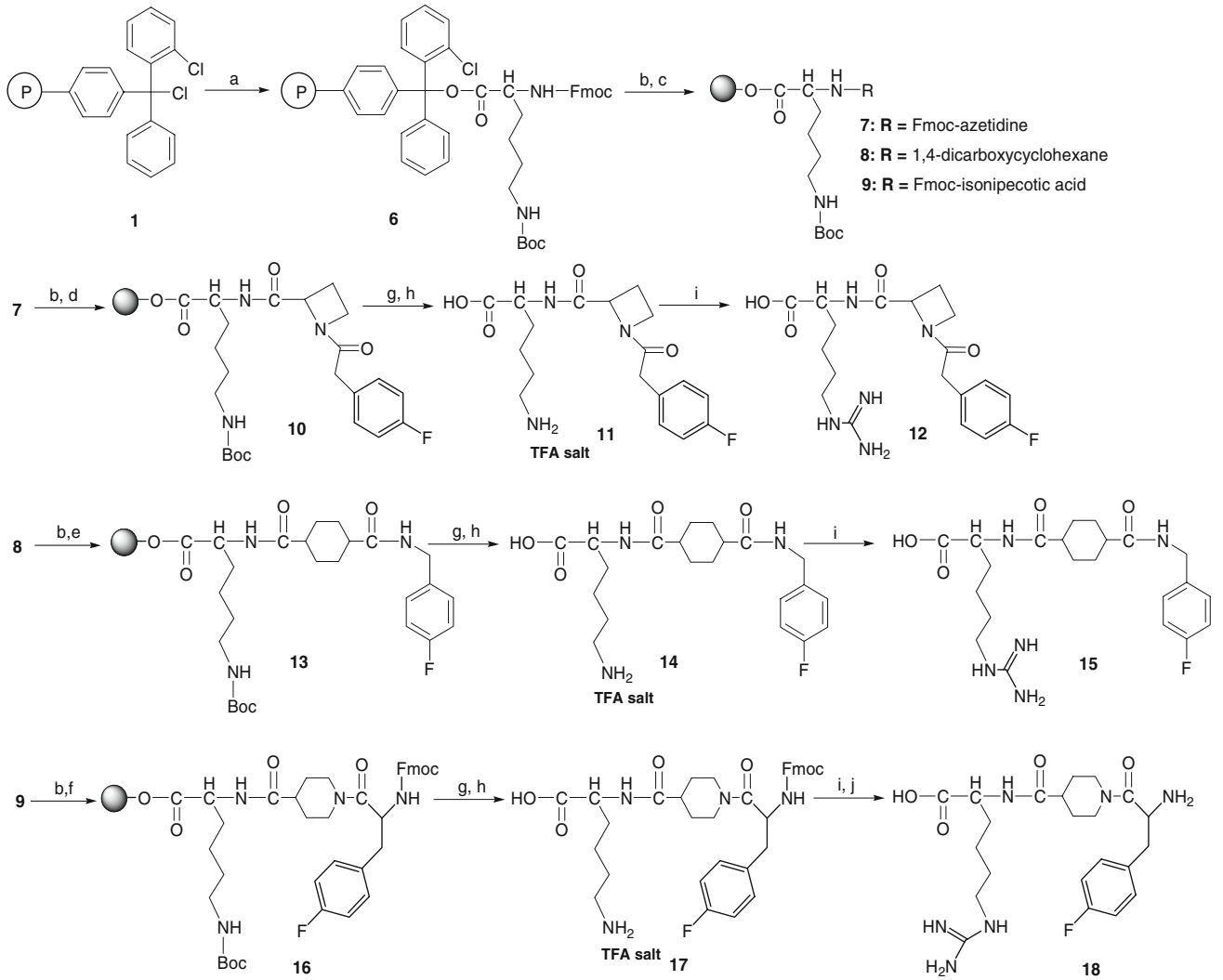
Data for compound **12**: ^{1}H-NMR (400 MHz; CD_3OD) $\delta_{ppm} = 7.54–7.07$ (m, 4H), 4.92–4.89 (m, 1H), 4.71–4.67 (t, 1H), 4.28–4.09 (t, 2H), 3.8 (s, 2H), 2.67 (t, 2H), 2.28 (m, 2H), 1.86 (m, 1H), 1.54 (m, 2H), 1.42 (m, 2H, H-6); ESI-MS, *m*/*z*, 408.02 (M^+), 409.03 (M + 1).

Data for compound **18**: ^{1}H-NMR ($CDCl_3$) $\delta_{ppm} = 7.26–6.98$ (m, 4H), 4.41 (t, 1H), 3.91–3.88 (m, 2H,), 3.13–3.01 (m, 6H), 2.62 (t, 2H), 2.38–2.29 (m, 4H), 1.91 (m, 4H), 1.73–1.59 (m, 2H), 1.43 (m, 2H); ESI-MS, *m*/*z*, 465.46 (M + 1), 466.38 (M + 2).

Results and discussion

Synthesis of mimetics

The synthesis of compound **5** was accomplished using a combination of solid and liquid phase organic synthesis. Attachment of the first amino acid Fmoc-Phe(F)-OH to the 2-chlorotritylchloride resin (Barlos et al. 1989) was achieved by a simple, fast and racemization-free reaction using a solution of DIPEA in DCM at RT. Removal of Fmoc protecting group using piperidine/DMF (1:4), followed by the coupling of Fmoc-Lys(Boc)-OH in the presence of activators HOBt/DIC, led to the fully protected dipeptide **3**. Cleavage of **3** from the resin with HFIP/DCM (3:7) and coupling with *N*-benzyl-5-amino-indole, in the presence of HOBt/DCC/DIPEA, afforded derivative **4**. Finally, deprotection of Boc- and Fmoc-protecting groups with TFA and piperidine solutions in DCM, respectively, furnished the final product **5** in high yield (overall 70% based on the calculated substitution of resin **2**) (Scheme 1). A similar methodology was applied for the synthesized compounds **12**, **15**, **18** following the general principle of solid phase organic synthesis-repeated cycles of coupling and deprotection. In particular, 2-chlorotritylchloride resin bound analogs **10**, **13**, **16** were synthesized using Fmoc methodology. These compounds bear a variety of scaffolds such as piperidine, azetidine and cyclohexane. Cleavage of fully protected compounds from the resin followed by selective deprotection of Boc group in acidic conditions (TFA/DCM), in the presence of scavengers, afforded **11**, **14**, **17**. Facile transformation of the amino to guanidino group of **11**, **14**, **17** using 1*H*-pyrazole-1-carboxamide hydrochloride (Bernatowicz et al. 1992) in DMF/DIPEA and subsequent Fmoc deprotection of the isonipecotic derivative led to **12**, **15** and **18**, respectively, in high yields (>80%) (Scheme 2). Purification of synthetic compounds was performed by preparative RP-HPLC using C_{18} column as stationary phase and UV peak detection. The purity of the lyophilized target final products **5** and **12**, **15**, **18** was determined by analytical RP-HPLC, followed by UV detection at 254 nm. Separations were achieved with a stepped linear gradient of acetonitrile (0.08% TFA) in water (0.08% TFA) and identification by ESI-MS and NMR.



Scheme 2 Reagents and conditions: **a** Fmoc-Lys(Boc)-OH, DIPEA, DCM, 1 h; **b** piperidine/DMF (1:5), 30 min; **c** *N*-Fmoc-azetidine or 1,4-dicarboxycyclohexane or Fmoc-isonipecotic acid, HOBt/DIC, DMF, 3 h; **d** 4-fluoro-phenylacetic acid, HOBt/DIC, DMF, 2 h; **e** 4-fluoro-benzylamine HOBt/DIC, DMF, 3 h; **f** *N*-Fmoc-4-fluoro-Phe-OH, HOBt/DIC, DMF, 3 h; **g** HFIP/DCM (3:7), 2 h; **h** TFA/DCM (1:1), TES, 1 h; **i** 1*H*-pyrazole-1-carboxamidine, DIPEA, DMF, 12 h; **j** piperidine/DCM (1:4), 2 h

Effects of mimetics on PAR_1-mediated calcium signaling

The ability of compound **5** to block PAR_1 activation was tested in the routine HEK cell calcium signaling assay in which the concentration of calcium in the incubation buffer is 5 mM (Kawabata et al. 1999). As shown in Fig. 1, pretreatment of HEK cells with compound **5** was able to block the calcium signal triggered either by thrombin or by the PAR_1-selective agonist, TFLLR-NH_2. The data shown in Fig. 1 were obtained from freshly prepared stock solutions of compound **5** (7–9 mM) dissolved in DMSO. It is important to point out for future work that when the stock solution was allowed to stand for several days in the refrigerator, compound **5** on its own (25–200 μM) caused a PAR-independent elevation of the steady-state intracellular calcium concentration that could be elevated further by TFLLR-NH_2 or thrombin action. In this setting, concentrations of compound **5** at 100 μM still completely blocked the ability of either thrombin or TFLLR-NH_2 to elevate intracellular calcium further, via PAR_1. At lower concentrations (5–10 μM), where compound **5** had little or no effect on the baseline concentration of intracellular calcium, it was still able to block thrombin-mediated elevations of intracellular calcium via PAR_1. The mechanism whereby these compound **5** stock solutions maintained in the refrigerator caused an elevation of basal intracellular calcium at concentrations greater than 25 μM was not evaluated further, except to verify that the effect was still present in PAR_1–PAR_2-desensitized HEK cells, indicating an action independent of PARs. In contrast, compound **15** on its own had no effect on the resting level of intracellular

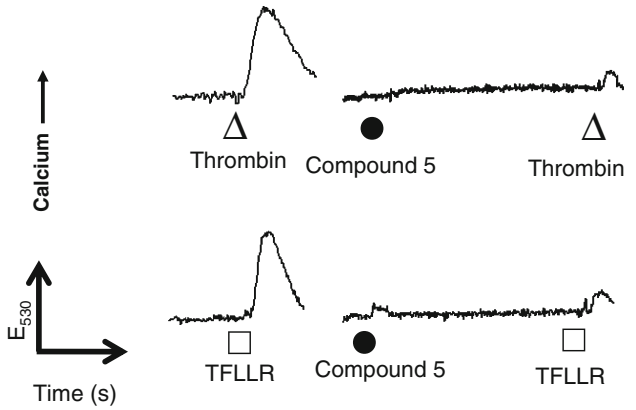


Fig. 1 Inhibition of PAR_1 calcium signaling by Compound **5** in HEK cells. The figure shows the increase in HEK cell intracellular calcium signaling (E_{530}: upward deflection, *arrow*) caused by the activation of PAR_1 by either thrombin (1 U/ml, *open triangle*, upper tracings) or the PAR_1-activating peptide (TFLLR-amide, 10 μM, *open square*, lower tracings) either without (left-hand tracings) or after (right-hand tracings) pretreatment of the cells with compound **5** (100 μM, *closed circle*). The directions of fluorescence (E_{530}) and time (seconds) are shown by the *inset*. The *arrows* indicate an upward deflection of 1 cm and a time span of 2 min, respectively

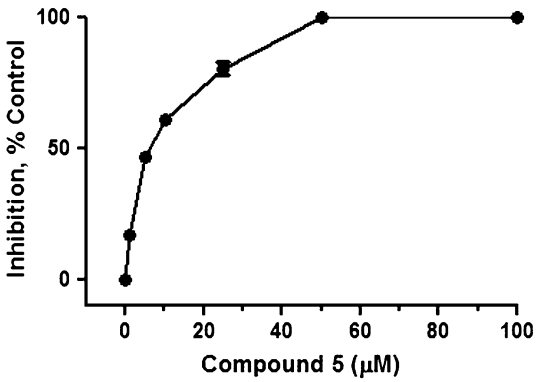


Fig. 2 Concentration–inhibition curve for blocking thrombin-mediated PAR_1 calcium signaling in HEK cells by compound **5**. Cells were pretreated with increasing concentrations of compound **5** and the calcium signal caused by thrombin (0.5 U/ml) via PAR_1 activation was monitored. The inhibition of calcium signaling was expressed as a percentage (% control) relative to the signal observed in cells that had not been treated with compound **5**. The *error bars* shown for the data obtained at 25 μM compound **5** are representative of the precision of the measurements. The IC_{50} for compound **5** was about 5 μM

calcium. Notwithstanding, like compound **5**, compound **15** (25–200 μM) was able to reduce PAR_1-mediated elevations in calcium caused by either thrombin (0.5 U/ml; 5 nM) or TFLLR-NH_2 (10 μM). The concentration–inhibition curve for the ability of compound **5** to prevent thrombin (0.5 U/ml)-triggered PAR_1 calcium signaling is shown in Fig. 2. The IC_{50} for this inhibitory action of compound **5** was about 5 μM. In this regard, the potency of compound **15** to block the activation of PAR_1 by thrombin was lower than that of compound **5**, because at 25 μM, compound **15** only partially blocked the thrombin/PAR_1-mediated calcium response, whereas compound **5** blocked the response to thrombin by abut 80% at this concentration (Fig. 2). This action of compound **5** was PAR-selective, because at a concentration of 25 μM under the same conditions where thrombin action was blocked, the PAR_1 antagonist did not affect the ability of trypsin to trigger a calcium signal via PAR_2 in the HEK cells (data not shown).

Structure and PAR_1-targeted action of mimetics

Herein, the design of non-peptide mimetics that can interact with and block PAR_1 activation was based on the current knowledge of the interactions of the tethered ligand with the thrombin receptor. Given that the cyclic analog of SFLLR (Adang et al. 1994; Moore et al. 1995) observed by NMR is probably the bioactive species, along with the observation that certain cyclic peptide derivatives are biologically active, we were prompted to design molecules that mimic the conformation adopted by SFLLR. This cyclic conformation brings the Phenylalanine and Arginine residues in close proximity. We therefore hypothesized that replacement of the aliphatic residues of the active peptide core with different scaffolds would yield novel PAR_1-targeted compounds.

In this report, according to this hypothesis and the structure-activity profile of the native pentapeptide SFLLR, different scaffolds (piperidine, azetidine, cyclohexane, indole) were utilized providing accordingly either structural flexibility or rigidity. Furthermore, these templates were selected due to the spatial requirements for displaying the key substituents in a suitable conformation and offered favorable synthetic considerations for their attachment. Ultimately, the mimetics we have prepared could be of value for the development of PAR_1 antagonists in addition to those that have been developed using a variety of other approaches (Andrade-Gordon et al. 1999; Zhang et al. 2003; Clasby et al. 2006).

Conclusions

This study describes the synthesis and biological evaluation of thrombin receptor mimetics bearing the essential phenylalanine/arginine pharmacophore groups of SFLLR on four different templates (piperidine, azetidine, cyclohexane and indole). Two of the templates yielded compounds (**5** and **15**) that will be able to block thrombin activation of PAR_1 and that will be of value for further optimization of non-peptide antagonists of PAR_1. The piperidine and azetidine scaffolds did not work as well as cyclohexane and indole presumably because these

scaffolds are rigid templates that do not allow a proper orientation of the pharmacophore groups to interact with the receptor. In particular, azetidine and piperidine are small rigid moieties in contrast to the flexible cyclohexane ring which can adopt both chair and boat conformations. Indole is also a larger scaffold compared to azetidine and piperidine which bear pharmacophores at the right distance for triggering activity.

Acknowledgments This research project is co-financed by EU-European Social Fund (75%) and the Greek Ministry of Development-GSRT (25%) with ancillary support from the Canadian Institutes of Health Research (CIHR: MDH).

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