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Synthesis and pharmacological evaluation of peptide-mimetic protease-activated receptor-1 antagonists containing novel heterocyclic scaffolds

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Abstract—Protease-activated receptor-1 (PAR-1) is a G-coupled receptor activated by α-thrombin and other proteases. In this paper we describe the synthesis and the pharmacological evaluation of novel peptide-mimetic antagonists (compounds **1**–**16**) characterized by the presence of new heterocyclic nuclei such as 2-methyl-indole (5- and 6-substituted) and 1,4-benzodiazepine moiety. The new derivatives, tested in order to evaluate their antagonist potency by using human platelet aggregation induced by PAR-1AP, resulted in some cases (compounds **1** and **4**) more potent than the reference. The compounds, tested on aortic rings, confirmed the results obtained in the aggregation assay.

1. Introduction

α-Thrombin is involved in many physiological functions such as coagulation, platelet aggregation, lymphocyte mitosis, monocyte chemotaxis, and endothelial cell proliferation. Many of these cellular effects are mediated activating the cell-surface receptor PAR-1, which is also known as thrombin receptor.[1–3] It belongs to the protease-activated receptors (PARs), a family of four G-protein-coupled receptors that share a common intriguing activation mechanism. A serine protease (α-thrombin, trypsin, tryptase, factor Xa) cleaves a specific site unmasking a new N-terminus (SFLLRN, in the case of PAR-1), which acts as a 'tethered ligand' that, internally, binds to the proximal heptahelical segment eliciting G-protein-coupled transmembrane signaling and cellular activation.[4] Studies with high affinity peptidic and peptide-mimetic agonists have demonstrated that PAR-1 is the major receptor responsible for mediating platelet aggregation, cell proliferation, inflammatory responses, and neurodegeneration.[5] α-Thrombin, by means of PAR-1 activation, might be, also, mechanistically involved in regulating hepatic fibrogenesis;[6] moreover many studies support the notion that PAR-1 plays a pivotal role in angiogenesis.[7] This means that this receptor is an attractive drug discovery target for the possible treatment of various disorders such as thrombosis, restenosis, atherosclerosis, inflammation, cancer metastasis, and stroke.[8] Design and preparation of peptide-mimetics or small organic molecules with PAR-1 antagonist properties have been very challenging since the tethered ligand-binding mechanism is energetically preferred; moreover very little information about conformation of the receptor is available and this means that a highly empirical approach has been used so far. Extensive structure–activity relationship studies have been performed providing a basic understanding of the peptide ligand side-chain structural requirements and their relative tolerances with regard to their interactions with the human thrombin receptor.[1,9] A 'three-point model', constituted by the ammonium group, the center of the benzene ring (Phe residue), and the central carbon of the guanidine group (Arg residue), has been used in conjunction with different molecular templates such as benzene, naphthalene, benzimidazole, and indole to design candidate peptide-mimetic structures.[8,9d,10] one of the most favoured template has been 6-aminoindole

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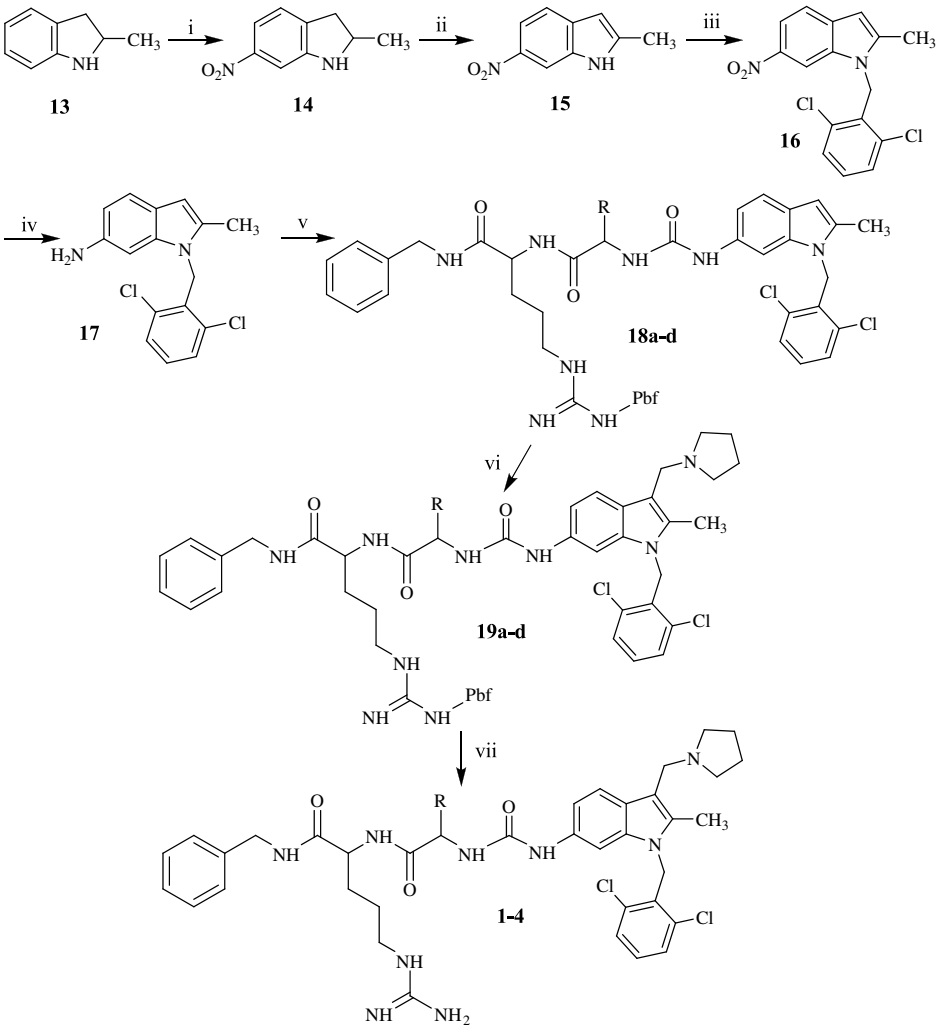
because it met the spatial requirements for displaying the three-key substituents in the correct three-dimensional configuration. Introduction of this scaffold in peptide-mimetic structures led to some active compounds that we have used as prototypes for developing our synthetic and pharmacological study.[8,10] Herein, we describe the synthesis and the pharmacological evaluation of novel heterocycle-based peptide-mimetic antagonists of PAR-1 (**1**–**16**) where the 6-aminoindole moiety has been displaced with the 2-methyl-6-aminoindole (compounds **1**–**4**) and 2-methyl-5-aminoindole (compounds **5**–**8**) templates. This substitution has been designed in order to verify if the introduction of the methyl group in position 2 could lead to a stronger interaction of the ammonium group (pyrrolidine) with the receptor even in the case of the 5-aminoindole nucleus which did not satisfy the desired geometry in previously reported series of compounds.[11] Successively, the use of 1,4-benzodiazepinic nucleus, a well-studied traditional pharmacophoric scaffold, has also been investigated (compounds **9**–**16**). Novel templates have been linked by an ureido motif to several aromatic unconventional amino acids such as *p*-methoxy-phenylalanine, 1-naphtylalanine, 2-naphtylalanine, and 3,4-difluoro-phenylalanine. All the synthesized compounds have been tested by using specific pharmacological assays to assess their ability to inhibit PAR-1AP (SFLLRN-NH_2) induced platelet aggregation.

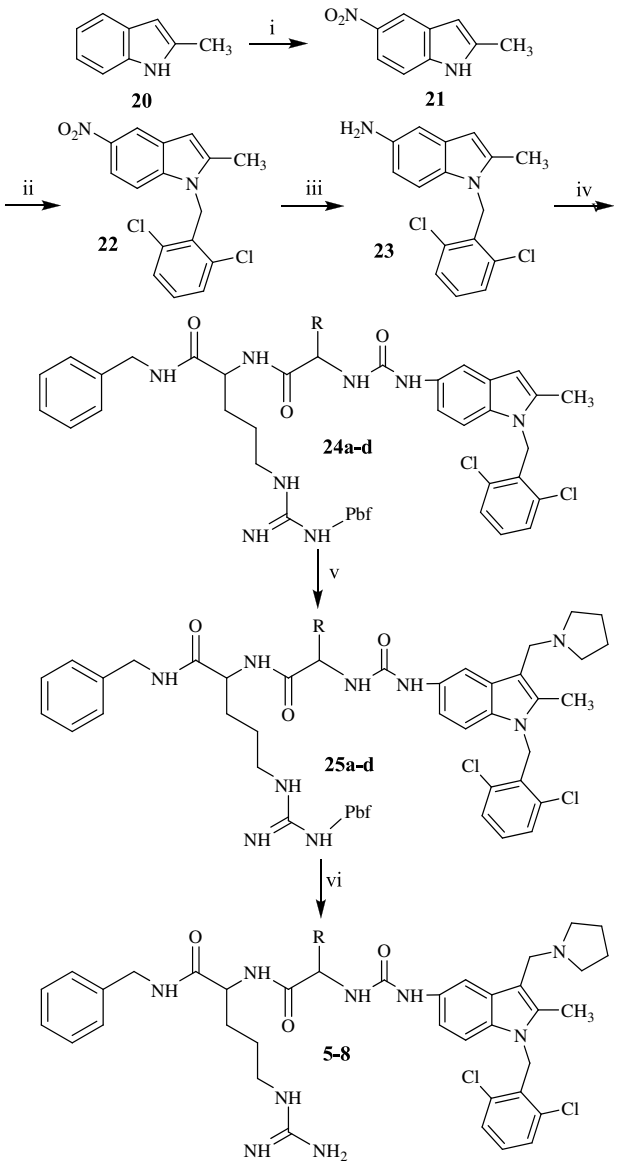
2. Chemistry

Compounds **1**–**4** were prepared according to the method of Andrade-Gordon et al.,[8a] ad hoc modified, via a convergent solution-phase method which requires a block procedure synthesis as shown in Scheme 1. The commercially available 2-methyl-2,3-dihydro-1*H*-indole **13** was treated with concentrated HNO_3/H_2SO_4 affording 6-nitro derivative **14**. The following oxidation with tetrachloro-1,4-benzoquinone in xylene gave 2-methyl-6-nitroindole **15**, which was treated with cesium carbonate and 2,6-dichlorobenzylbromide. Reduction of **16** with Pd/C and $NaBH_4$ in methanol furnished the 6-amino-indole derivative **17**, which was treated with *p*-nitrophe-

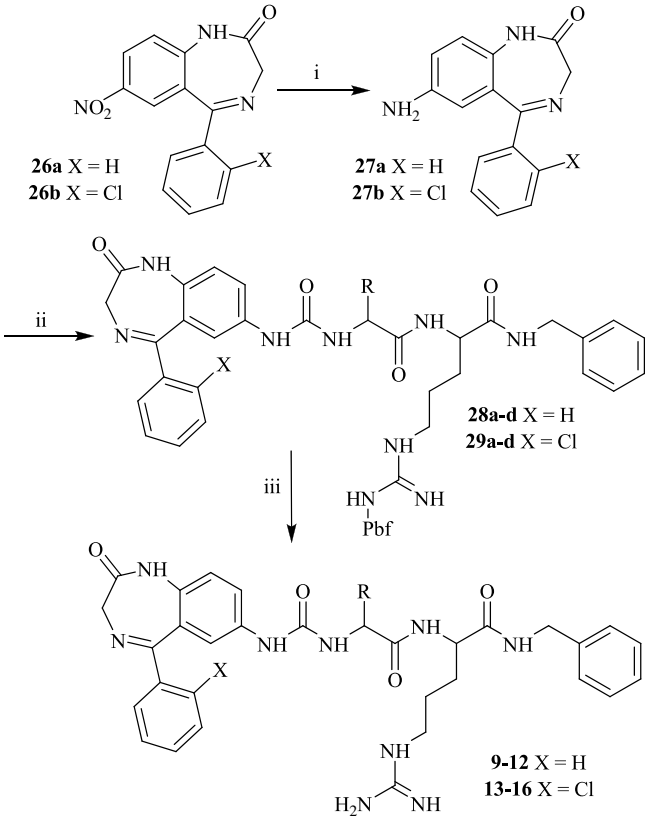


Scheme 1. Synthetic procedure for compounds **1**–**4**. Reagents and conditions: (i) HNO_3/H_2SO_4; (ii) tetrachloro-*p*-benzoquinone, xylene; (iii) 2,6-dichlorobenzyl bromide, Cs_2CO_3, DMF; (iv) $NaBH_4$, 10% Pd/C, CH_3OH/THF; (v) *p*-nitrophenylchloroformate, NMM, DCM anhydrous, −20 °C, 30 min then H-X-Arg(Pbf)-NH-CH_2-C_6H_5, NMM, rt, 16 h; (vi) pyrrolidine, CH_2O, CH_3COOH; (vii) CF_3COOH, rt, 2 h.

nylchloroformate and *N*-methylmorpholine (NMM) at −20 °C in dichloromethane (DCM). The addition of the opportune H-X-Arg(Pbf)-CO-NH-CH_2-C_6H_5, synthesized by standard solution method,[8a] afforded the indole-urea derivatives **18a–d**. Mannich reaction with pyrrolidine and formaldehyde in glacial acetic acid and cleavage of 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl (Pbf) group from the intermediates **19a–d** by treatment with trifluoroacetic acid (TFA) for 2 h at room temperature gave the final products **1–4**. Compounds **5–8** were prepared according to the method reported in Scheme 2. Nitration of commercially available 2-methyl-indole **20** with $NaNO_3$ in H_2SO_4 furnished the intermediate **21**. The purified derivative **21** was reacted with 2,6-dichlorobenzylbromide in presence of cesium carbonate in *N*,*N*-dimethylformamide (DMF). Nitro reduction of **22** with Pd/C and $NaBH_4$ in methanol provided the 5-amino-indole derivative **23**, which was reacted with the opportune H-X-Arg(Pbf)-CO-NH-CH_2-C_6H_5 in presence of *p*-nitrophenylchloroformate. The urea derivatives **24a–d** were treated with pyrrolidine and formaldehyde in glacial acetic acid affording the intermediates **25a–d**. Pbf cleavage by means of TFA for 2 h at room temperature provided the final compounds **5–8**. 1,4-Benzodiazepinic derivatives (**9–16**) have been synthesized following the procedure reported in Scheme 3. Nitrazepam **26a** or clonazepam **26b**[12] were hydrogenated to the corresponding amino derivatives **27a** and **27b**. Reaction with *p*-nitrophenylchloroformate and NMM in anhydrous DCM followed by the addition of the opportune H-X-Arg(Pbf)-CO-NH-CH_2-C_6H_5 furnished the ureidic derivatives **28a–d** and **29a–d**. Treatment with neat TFA to remove the Pbf-protecting group provided the final compounds **9–16**. All the intermediates were characterized by NMR and ESI mass spectrometry. Purification of each final product was obtained by reverse-phase high performance liquid chromatography (RP-HPLC) to greater than 98% purity. All the new compounds gave satisfactory elemental analyses and were characterized by NMR and ESI mass spectrometry. ^{1}H NMR and MS data for all final compounds were consistent with the proposed structures (see Tables 1 and 2).

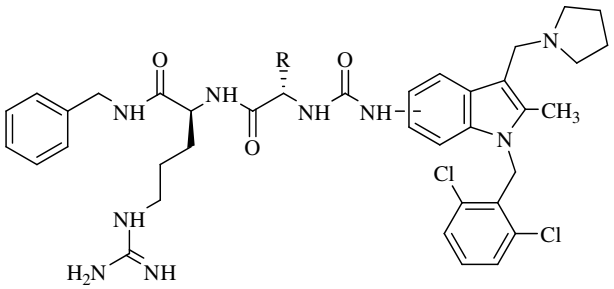


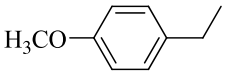
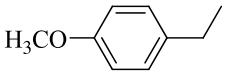
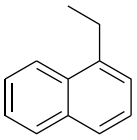
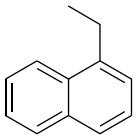
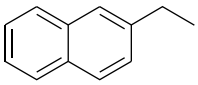
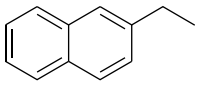
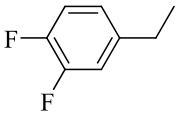
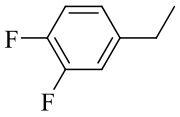
Scheme 2. Synthetic procedure for compounds **5–8**. Reagents and conditions: (i) $NaNO_3/H_2SO_4$, 0 °C; (ii) 2,6-dichlorobenzyl bromide, Cs_2CO_3, DMF; (iii) $NaBH_4$, 10% Pd/C, CH_3OH/THF; (iv) *p*-nitrophenylchloroformate, NMM, DCM anhydrous, −20 °C, 30 min then H-X-Arg(Pbf)-NH-CH_2-C_6H_5, NMM, rt, 16 h; (v) pyrrolidine, CH_2O, CH_3COOH; (vi) CF_3COOH, rt, 2 h.



Scheme 3. Synthetic procedure for compounds **9–16**. Reagents and conditions: (i) H_2 (2 atm), 10% Pd/C, CH_3OH, 45 min; (ii) *p*-nitrophenylchloroformate, NMM, DCM anhydrous, −20 °C, 30 min then H-X-Arg(Pbf)-NH-CH_2-C_6H_5, rt, 16 h; (iii) CF_3COOH, rt, 2 h.

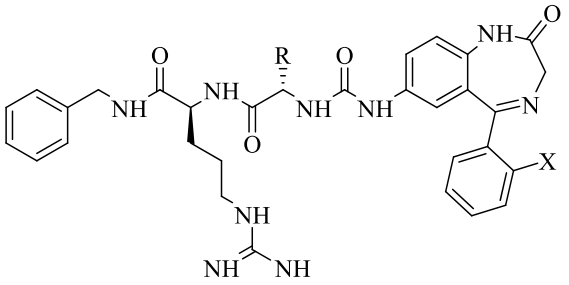
Table 1. Indole-based peptide-mimetics as PAR-1 antagonists

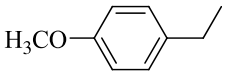
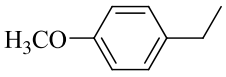
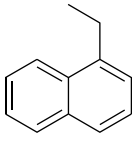
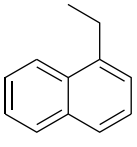
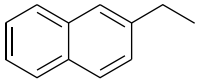
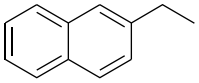
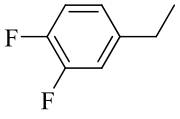
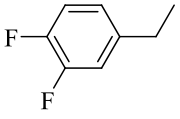


Compound	P[a]	R	Compound	P[a]	R
1	6		**5**	5	
2	6		**6**	5	
3	6		**7**	5	
4	6		**8**	5	

[a] P denotes the position of indole ring attaching to urea linkage.

Table 2. Benzodiazepine-based peptide-mimetics as PAR-1 antagonists



Compound	R	X	Compound	R	X
9		H	**13**		Cl
10		H	**14**		Cl
11		H	**15**		Cl
12		H	**16**		Cl

3. Results and discussion

The pharmacological activity was assessed by using human platelet aggregation induced by PAR-1AP in order to test the antagonist potency. Among the newly synthesized derivatives, screened and reported in Figure 1, compounds **1** and **4** resulted more potent than the reference compound RWJ54003, while compound **3** presented a similar activity. A full dose–response analysis was performed in order to compare the relative potency of compounds **1**, **3**, and **4** versus the reference compound. As reported in Figure 2 the dose response generated by compounds **1** and **4** as opposite to the reference compound is rather steep in their slope suggesting a different kinetic or interaction with the receptor. Furthermore the two compounds did not differ at IC_{50} level, while they show a different profile at the higher concentrations. Indeed the IC_{75} analysis gave 0.1 μM for the reference compound, 0.8 μM for compound **3**, 0.02 and 0.05 μM for compounds **1** and **4**, respectively. To confirm the PAR-1 antagonist activity of these compounds, aortic rings were incubated with the test compounds (10–100 μM) or vehicle; after 15 min aortic rings were contracted with PAR-1AP (SFLLRN-NH_2) in cumulative manner (1×10^{-7}–3×10^{-3} M). Data were calculated in percent of inhibition ± SEM. In Figure 3 are reported the compounds displaying an inhibitory effect. Particularly, compounds **1** and **4** showed an enhanced inhibitory activity when compared to RWJ54003, while a similar inhibitory activity was displayed by compound **3**. In particular the enhanced inhibitory activity was evident at the higher dose of PAR-1AP used. Indeed, the maximal contraction achieved with PAR-1AP (212 ± 33 dyne/mg) was signif-

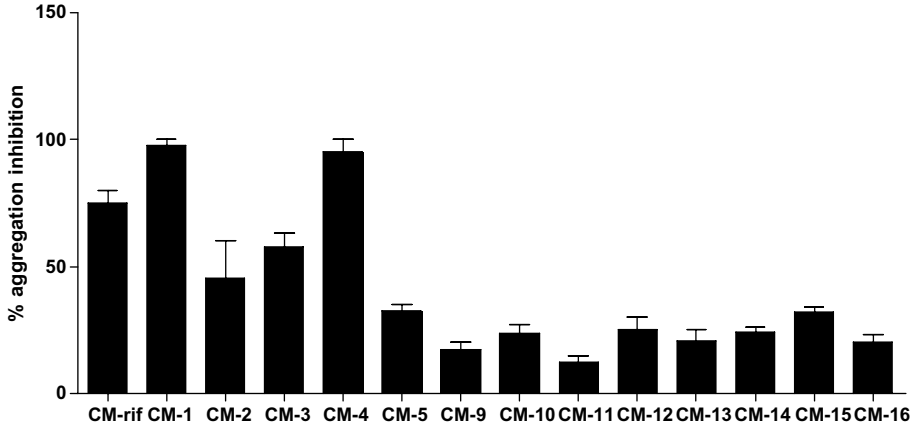


Figure 1. Effect of the tested compounds and the reference compound RWJ54003 on human platelet aggregation induced by PAR-1AP (SFLLRN-NH_2). The platelet count was adjusted to 2.5×10^8/mL and the threshold for PAR-1AP-induced human platelet aggregation was obtained. The influence of the tested compounds (10^{-7} M) on PAR-1AP (10^{-6} M)-induced human platelet aggregation was determined. Data were calculated in percent of inhibition ± SEM. $^*p < 0.01$ versus CM rif.

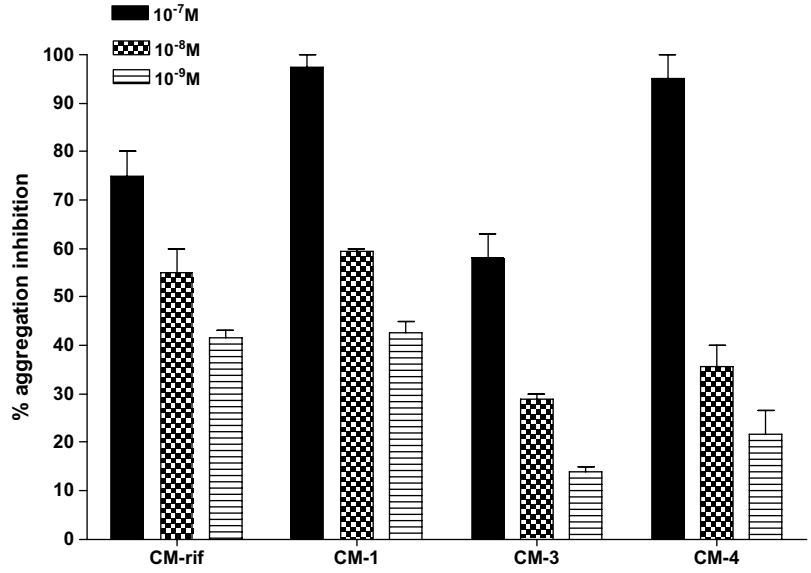


Figure 2. Human platelets were incubated with different concentrations (10^{-7}–10^{-9} M) of the reference compound RW54003 or the more active screened compounds e.g. **CM-1**, **CM-3**, **CM-4**; following 10 min of incubation PAR-1AP-induced human platelet aggregation was determined. Data were calculated in percent of inhibition ± SEM.

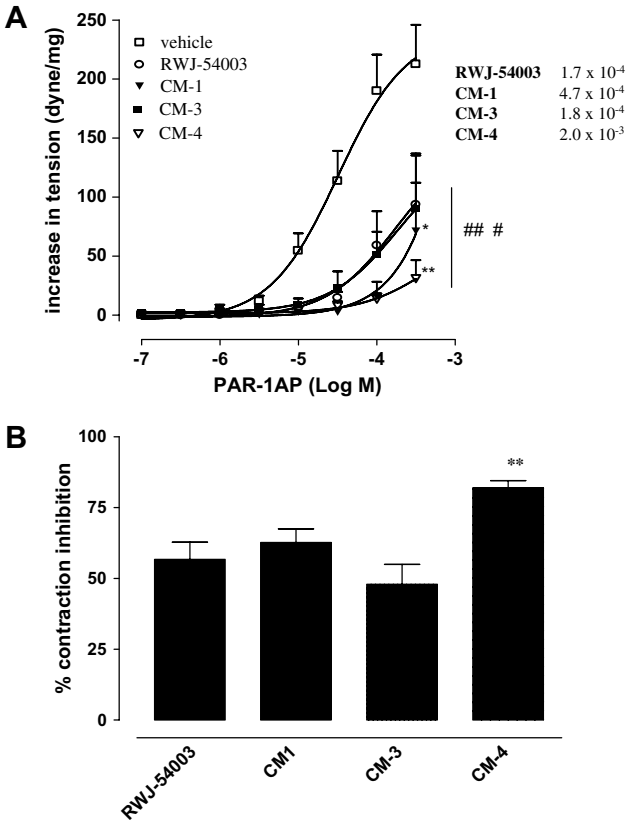


Figure 3. Aortic rings were incubated with the tested compounds (10–100 μM) or vehicle, after 15 min aortic rings were contracted with PAR-1AP in a cumulative manner (1×10^{-7}–3×10^{-3} M). Panel (A) represents the concentration–response curves to PAR-1AP. $^{*}p < 0.01$, $^{**}p < 0.001$ versus RWJ-54003; $^{\#\#\#}p < 0.001$ versus vehicle; panel (B) represents data expressed as percent of inhibition ± SEM. $^{**}p < 0.001$ versus RWJ-54003.

icantly reduced to 94 ± 18 dyne/mg ($p < 0.01$) by RWJ-54003 while **4** reduced the maximal contraction to 31 ± 16 dyne/mg ($p < 0.01$).

Our results are in accordance with those obtained with the previously reported derivatives in which the same unnatural residues furnished the best affinity and activity.[8] Compound **1**, particularly, differs from the reference compound RWJ54003 only because of the presence of a methyl group in position 2 of the 6-aminoindole moiety. This little structural modification shifts the biological activity towards a more active derivative as compared to the reference compound suggesting that the presence of a methyl group could improve the inhibitory effect. This improved affinity could be due to a stronger interaction between the pyrrolidine nitrogen and a negatively charged group of the receptor binding site and/or a modification of 2,6-dichlorobenzyl moiety bond angle that could allow an enhanced binding by virtue of additional favorable interactions. The 5-aminoindole derivatives (compounds **5**–**8**) were found inactive or weak inhibitors of PAR-1AP-induced platelet aggregation (compound **5**, Fig. 1) and PAR-1AP-induced aorta ring contraction (compounds **5** and **6**, data not shown) confirming that the shift of the pseudopeptidic moiety to position 5 of the indole nucleus causes a dramatic change in the three-dimensional structure of the scaffold hindering the molecular recognition needed for the biological response. The substitution of the 6-amino-indole nucleus with 1,4-benzodiazepinone scaffold afforded compounds **9**–**16** that display just a weak inhibitory effect (Fig. 1). The introduction of the 2-chlorophenyl group, in place of a plain phenyl ring, on the benzodiazepinic moiety (compounds **13**–**16**) has been designed in order to better mimic the substituent present on the indole nitrogen of compounds **1**–**8**. Nonetheless, just compound **15** has shown a slight, but not significant, increasing of aggregation percentage. These data prompt us to hypothesize that the dramatic lack of activity, registered for this series of compounds, could be primarily addressed to the lack of the second basic center (such as pyrrolidine nitrogen present in the amino-indole derivatives).

4. Conclusion

Our study describes the synthesis and the pharmacological evaluation of a series of peptide-mimetic compounds as novel PAR-1 antagonists. The 6-amino-indole derivatives furnished the better results in both the performed functional assays. Pharmacological evaluation of the synthesized derivatives indicates compounds **1** and **4** as more active than the reference RWJ54003 in the platelet aggregation inhibition. Our results support the observation that PAR-1 antagonists could have a significant utility in the treatment of thrombotic disorders such as platelet-driven arterial thrombosis. Moreover, a PAR-1 antagonist, compared to a direct thrombin inhibitor, should act as a specific cellular activator of thrombin, not affecting its enzymatic activity (e.g. fibrin generation); this means that such an agent is likely to have less bleeding liability than the currently existing antithrombotic drugs.

5. Experimental

5.1. Materials and methods

All solvents were purchased from Carlo Erba (Rodano, Milan, Italy). Extraction solvents were dried over sodium sulfate. Solvents used for reactions were dried over 3 Å molecular sieves. All solvents were filtered and degassed prior to the use. Reagent-grade materials were purchased from Bachem (Bubendorf, Switzerland) and from Aldrich (Milan, Italy) and were used without further purification. Thin-layer chromatography was performed on precoated silica gel Kieselgel 60F254 (Merck, A.G., Darmstadt, Germany) plates. The compounds were detected on thin-layer chromatography plates by UV light or ninhydrin. Molecular weights of final peptide-mimetic derivatives were assessed by electrospray ionization mass spectrometry (ESI/MS) performed on a LCQ Thermoquest-Ion trap mass spectrometer. Where analyses are indicated only by the symbols of the elements, results obtained are within ±0.4% of the theoretical values. The ^{1}H NMR spectra

were recorded on a Bruker AMX-500 spectrometer. Chemical shifts are reported in ppm using Me_4Si as an internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), and m (multiplet). Reverse-phase purification was routinely performed on a Waters Delta Prep 4000 system equipped with a Waters 484 multi-wavelength detector on a Vydac C_{18} silica (15–20 μm, 22 × 5000 mm) high-performance liquid chromatography (HPLC) column. The operational flow rate was 30 mL/min. Homogeneity of the products was assessed by analytical reverse-phase HPLC using a Vydac C_{18} column (5 μm, 4.6 × 250 mm) employing the following conditions: eluent A, 0.05% TFA (v/v) in water; eluent B, 0.05% TFA (v/v) in acetonitrile; gradient 0–70% B over 25 min, UV detection at 220 nm, and flow rate 1 mL/min. The column was connected to a Rheodyne model 7725 injector, a Waters 600 HPLC system, a Waters 486 tunable absorbance detector set to 220 nm, and a Waters 746 chart recorder.

5.2. Synthesis of 2-methyl-5 or 6-amino-indole derivatives (compounds 1–8)

5.2.1. 2-Methyl-6-nitro-2,3-dihydro-1*H*-indole (14). 2-Methyl-2,3-dihydro-1*H*-indole (**13**, 11.7 g, 0.088 mol) was added slowly to 100 mL of concentrated sulfuric acid maintained at a temperature of 5 °C; 4.4 mL of concentrated nitric acid was then added dropwise at 0 °C. The reaction mixture was stirred at this temperature for 1 h, then poured into 100 mL of saturated solution Na_2CO_3, and the product was extracted with diethyl ether. The organic layer was dried over Na_2SO_4 and the solvent was removed in vacuo. The residue was purified on a silica gel column using *n*-hexane/diethyl ether (9:1) as eluent and the pure product **14** was obtained as a yellow solid: 10.7 g (68%). MS/ESI (+), *m*/*z*: 179 $[M+H]^+$. ^1H NMR ($CDCl_3$) δ 1.31 (d, 3H), 2.66 (m, 1H), 3.18 (m, 1H), 4.11 (m, 1H), 7.11 (d, 1H), 7.35 (s, 1H), 7.56 (d, 1H).

5.2.2. 2-Methyl-6-nitro-1*H*-indole (15). 2-Methyl-6-nitro-2,3-dihydro-1*H*-indole (**14**, 6 g, 0.034 mol) and tetrachloro-1,4-benzoquinone (8.3 g, 0.034 mol) were dissolved in xylene (150 mL). The solution was stirred at 140 °C for 2 h and then left overnight at room temperature. The reaction mixture was alkalinized with 1 N NaOH, extracted with DCM, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified on silica gel column using a mixture of diethyl ether/*n*-hexane (5:5) as eluent and the pure product **15** was obtained as a yellow solid: yield 2 g (33%). MS/ESI (+), *m*/*z*: 177 $[M+H]^+$. ^1H NMR ($CDCl_3$) δ 2.52 (s, 3H), 6.35 (s, 1H), 7.51 (d, 1H), 7.98 (d, 1H), 8.25 (s, 1H).

5.2.3. 1-(2,6-Dichlorobenzyl)-2-methyl-6-nitro-1*H*-indole (16). To a solution of 2-methyl-6-nitro-1*H*-indole (**15**, 2 g, 0.014 mol) in DMF (50 mL) were added cesium carbonate (4.5 g, 0.014 mol) and 2,6-dichlorobenzylbromide (3.3 g, 0.014 mol). The solution was stirred at 60 °C for 1 h; then the reaction mixture was cooled, the solid was filtered off, and the filtrate was concentrated in vacuo. The product **16** was precipitated with diethyl ether as a yellow solid: yield 3.3 g (70%). MS/ESI (+), *m*/*z*: 336 $[M+H]^+$. ^1H NMR ($CDCl_3$) δ 2.43 (s, 3H), 5.59 (s, 2H), 6.36 (s, 1H), 7.26 (t, 1H), 7.37 (d, 2H), 7.47 (d, 1H), 7.90 (d, 1H), 8.10 (s, 1H).

5.2.4. 1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-ylamine (17). To a suspension of $NaBH_4$ (0.74 g, 0.02 mol) and 10% Pd/C (50 mg) in methanol (30 mL) was added a solution of 1-(2,6-dichlorobenzyl)-2-methyl-6-nitro-1*H*-indole (**16**, 3.3 g, 0.01 mol) in THF (100 mL). After 3 h the reaction mixture was filtered, the solvent was removed in vacuo, and the residue was dissolved in chloroform and washed with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The crude product **17** was purified on silica gel column using a mixture of ethyl acetate/*n*-hexane (6:4) and crystallized with diethyl ether: yield 2.2 g (72%). MS/ESI (+), *m*/*z*: 306 $[M+H]^+$. ^1H NMR ($CDCl_3$) δ 2.33 (s, 3H), 5.40 (s, 2H), 6.13 (s, 1H), 6.43 (s, 1H), 6.50 (d, 1H), 7.19 (t, 1H), 7.26 (d, 2H), 7.31 (d, 1H).

5.2.5. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-yl]-ureido}-3-(4-methoxyphenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (18a). 1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-ylamine (**17**, 0.3 g, 1 mmol) was dissolved in anhydrous DCM (50 mL). The mixture was cooled to −20 °C and *p*-nitrophenylchloroformate (0.24 g, 1.2 mmol) and NMM (0.22 mL, 2 mmol) were added. After 30 min H-Tyr(Me)-Arg(Pbf)-NH-CH_2-C_6H_5 (0.7 g, 1 mmol) was added and the reaction mixture was allowed to warm to room temperature and stirred for an additional 16 h. Then the solution was ice-bath cooled and the solid **18a** was filtered and let air-dry. The product was used without further purification: yield 0.73 g (73%). MS/ESI (+), *m*/*z*: 1025 $[M+H]^+$.

5.2.6. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-yl]-ureido}-3-naphthalen-1-yl-propionylamino)-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (18b). Starting from **17** and H-1-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **18b** was obtained following the synthetic procedure reported for compound **18a**: yield 0.83 g (80%). MS/ESI (+), *m*/*z*: 1045 $[M+H]^+$.

5.2.7. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-yl]-ureido}-3-naphthalen-2-yl-propionylamino)-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (18c). Starting from **17** and H-2-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **18c** was obtained following the synthetic procedure reported for compound **18a**: yield 0.78 g (75%). MS/ESI (+), *m*/*z*: 1045 $[M+H]^+$.

5.2.8. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-6-yl]-ureido}-3-(3,4-difluorophenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (18d). Starting from **17** and H-3,4-diF-Phe-Arg(Pbf)-NH-CH_2-C_6H_5, compound **18d** was obtained following the synthetic procedure re-

ported for compound **18a**: yield 0.75 g (73%). MS/ESI (+), *m/z*: 1031 $[M+H]^+$.

5.2.9. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-6-yl]-ureido}-3-(4-methoxyphenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (1). The indole-urea derivative **18a** (0.73 g, 0.7 mmol) was dissolved in glacial acetic acid (5 mL) and treated with a solution of pyrrolidine (0.15 mL, 1.75 mmol) and formaldehyde (0.15 mL, 1.75 mmol) and the mixture was stirred for 24 h at 60 °C. Then the solution was cooled and adjusted to pH 12 using 2 N KOH; the precipitated solid **19a** was filtered and let dry in vacuo furnishing 0.7 g of crude product that was dissolved in TFA (5 mL); the mixture was stirred for 2 h at room temperature. The solution was evaporated to dryness in vacuo and the residue was triturated with diethyl ether giving 0.6 g of crude product **1**, which was purified on a Vydac C_{18} silica (15–20 μm, 22 × 5000 mm) high performance liquid chromatography (HPLC) column. The product was eluted with a gradient of 0–70% B in 25 min at a flow rate of 30 mL/min using the following mobile phase: solvent A (water in 0.1% TFA) and solvent B (acetonitrile in 0.1 %TFA). MS/ESI (+), *m/z*: 855 $[M+H]^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (m, 3H), 1.80 (m, 3H), 2.0 (m, 2H), 2.38 (s, 3H), 2.76 (m, 1H), 2.90 (m, 4H), 3.08 (m, 3H), 3.66 (s, 3H), 4.20 (m, 4H), 4.44 (s, 2H), 5.50 (s, 2H), 6.73 (d, 2H), 7.06 (t, 2H), 7.14 (s, 1H), 7.22–7.49 (m, 10H). Anal. Calcd for $C_{45}H_{53}Cl_2N_9O_4$: C, 63.22; H, 6.25; N, 14.75. Found: C, 63.21; H, 6.23; N, 14.71.

5.2.10. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-6-yl]-ureido}-3-naphthalen-1-yl-propionylamino)-5-guanidino-pentanoic acid benzylamide (2). Starting from **19b**, compound **2** was obtained following the synthetic procedure reported for compound **1**: yield 0.75 g of crude product. MS/ESI (+), *m/z*: 875 $[M+H]^+$. ^{1}H NMR (DMSO-d_6) δ 1.59 (m, 6H), 1.79 (m, 2H), 2.40 (s, 3H), 2.70 (m, 4H), 2.90 (m, 2H), 3.19 (m, 1H), 3.47 (m, 3H), 4.44 (m, 3H), 4.50 (m, 1H), 5.11 (s, 2H), 6.70 (d, 2H), 7.01–7.10 (m, 6H), 7.13–7.69 (m, 10H). Anal. Calcd for $C_{48}H_{53}Cl_2N_9O_3$: C, 65.89; H, 6.11; N, 14.41. Found: C, 65.80; H, 6.13; N, 14.45.

5.2.11. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-6-yl]-ureido}-3-naphthalen-2-yl-propionylamino)-5-guanidino-pentanoic acid benzylamide (3). Starting from **19c**, compound **3** was obtained following the synthetic procedure reported for compound **1**: yield 0.69 g of crude product. MS/ESI (+), *m/z*: 875 $[M+H]^+$. ^{1}H NMR (DMSO-d_6) δ 1.43 (m, 3H), 1.85 (m, 3H), 2.30 (m, 4H), 2.47 (s, 3H), 2.55 (m, 2H), 3.04 (m, 1H), 3.50 (m, 3H), 4.37 (m, 3H), 4.56 (m, 1H), 5.21 (s, 2H), 6.75 (d, 2H), 6.95–7.14 (m, 6H), 7.28–7.68 (m, 10H). Anal. Calcd for $C_{48}H_{53}Cl_2N_9O_3$: C, 65.89; H, 6.11; N, 14.41. Found: C, 65.91; H, 6.08; N, 14.43.

5.2.12. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-6-yl]-ureido}-3-(3,4-difluorophenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (4). Starting from **19d**, compound **4** was obtained following the synthetic procedure reported for compound **1**: yield 0.65 g of crude product. MS/ESI (+), *m/z*: 861 $[M+H]^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (m, 8H), 2.35 (s, 3H), 2.85 (dd, 1H), 3.00 (m, 5H), 3.30 (m, 2H), 4.35 (m, 5H), 4.60 (m, 1H), 5.45 (s, 2H), 7.01–7.60 (m, 13H), 7.77 (s, 1H). Anal. Calcd for $C_{44}H_{49}Cl_2F_2N_9O_3$: C, 61.39; H, 5.74; N, 14.64. Found: C, 61.37; H, 5.72; N, 14.68.

5.2.13. 2-Methyl-5-nitro-3-pyrrolidin-1-ylmethyl-1*H*-indole (21). To an ice-cold solution of 2-methyl-1*H*-indole (**21**) (4 g, 0.03 mol) in sulfuric acid (25 mL) was added, dropwise, $NaNO_3$ (2.7 g) dissolved in sulfuric acid (25 mL). After the addition was completed, the reaction mixture was poured in ice and the obtained yellow solid **21** was filtered off, washed with water, and then let air-dry: yield 4.8 g (90%). MS/ESI (+), *m/z*: 177 $(M + H^+)$. ^{1}H NMR ($CDCl_3$) δ 2.45 (s, 3H), 6.39 (s, 1H), 7.29 (d, 1H), 8.00 (d, 1H), 8.45 (s, 1H).

5.2.14. 1-(2,6-Dichlorobenzyl)-2-methyl-5-nitro-1*H*-indole (22). To a solution of 2-methyl-5-nitro-1*H*-indole (**21**, 2.5 g, 0.014 mol) in DMF (50 mL) were added cesium carbonate (4.6 g, 0.014 mol) and 2,6-dichlorobenzylbromide (3.3 g, 0.014 mol). The solution was stirred at 60 °C for 1 h; then the reaction mixture was cooled, the solid was filtered, and the filtrate was concentrated in vacuo. The product **22** was precipitated with diethyl ether as a yellow solid: yield 2.1 g (45%). MS/ESI (+), *m/z*: 336 $(M + H^+)$. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H), 5.57 (s, 2H), 6.43 (s, 1H), 7.06 (d, 1H), 7.24 (t, 1H), 7.36 (d, 2H), 7.90 (d, 1H), 8.42 (s, 1H).

5.2.15. 1-(2,6-Dichlorobenzyl)-2-methyl-3-1*H*-indol-5-ylamine (23). To a suspension of $NaBH_4$ (0.47 g, 0.012 mol) and Pd/C (20 mg) in methanol (50 mL) was added a solution of 22 (2.1 g, 6.3 mmol) in THF (50 mL). After 3 h the reaction mixture was filtered, the solvent was removed in vacuo, and the residue was dissolved in chloroform and washed with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The crude product 23 was purified on silica gel column using a mixture of ethyl acetate/*n*-hexane (6:4) and crystallized with diethyl ether: yield 1.3 g (68%). MS/ESI (+), *m/z*: 306 $[M+H]^+$. ^{1}H NMR ($CDCl_3$) δ 2.49 (s, 3H), 5.50 (s, 2H), 6.20 (s, 1H), 6.40 (s, 1H), 6.55 (d, 1H), 7.10 (d, 1H), 7.26 (t, 1H), 7.37 (d, 2H).

5.2.16. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-1*H*-indol-5-yl]-ureido}-3-(4-methoxyphenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (24a). Starting from **23** and H-Tyr(Me)-Arg(Pbf)-NH-CH_2-C_6H_5 compound **24a** was obtained following the synthetic procedure reported for compound **18a**: yield 0.75 g (73%). MS/ESI (+), *m/z*: 1025 $[M+H]^+$.

5.2.17. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-5-yl]-ureido}-3-naphthalen-1-yl-propionylamino)-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (24b). Starting from **23** and H-1-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **24b** was obtained following the synthetic proce-

dure reported for compound **18a**: yield 0.80 g (77%). MS/ESI (+), *m*/*z*: 1045 $[M+H]^+$.

5.2.18. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1H-indol-5-yl]-ureido}-3-naphthalen-2-yl-propionylamino)-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (24c). Starting from **23** and H-2-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **24c** was obtained following the synthetic procedure reported for compound **18a**: yield 0.76 g (73%). MS/ESI (+), *m*/*z*: 1045 $[M+H]^+$.

5.2.19. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-1*H*-indol-5-yl]-ureido}-3-(3,4-difluorophenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (24d). Starting from **23** and H-3,4-diF-Phe-Arg(Pbf)-NH-CH_2-C_6H_5, compound **24d** was obtained following the synthetic procedure reported for compound **18a**: yield 0.74 g (72%). MS/ESI (+), *m*/*z*: 1031 $[M+H]^+$.

5.2.20. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-5-yl]-ureido}-3-(4-methoxyphenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (5). The indole-urea derivative **24a** (0.75 g, 0.7 mmol) was dissolved in glacial acetic acid (5 mL) and treated with a solution of pyrrolidine (0.15 mL, 1.75 mmol) and formaldehyde (0.15 mL, 1.75 mmol) and the mixture was stirred for 24 h at 60 °C. Then the solution was cooled and adjusted to pH 12 using 2 N KOH; the precipitated solid **25a** was filtered and let dry in vacuo furnishing 0.65 g of crude product that was dissolved in TFA (5 mL); the mixture was stirred for 2 h at room temperature. The solution was evaporated to dryness in vacuo and the residue was triturated with diethyl ether giving 0.6 g of crude product **5** which was purified by RP-HPLC using the same condition reported for compound **1**. MS/ESI (+), *m*/*z*: 855 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.45 (m, 6H), 1.88 (m, 2H), 2.25 (m, 4H), 2.30 (s, 3H), 2.65 (m, 2H), 2.88 (m, 1H), 3.08 (m, 1H), 3.50 (s, 2H), 3.60 (s, 3H), 4.22 (m, 3H), 4.92 (m, 1H), 5.50 (s, 2H), 6.73 (d, 2H), 6.80 (d, 1H), 7.14–7.49 (m, 12H). Anal. Calcd for $C_{45}H_{53}Cl_2N_9O_4$: C, 63.22; H, 6.25; N, 14.75. Found: C, 63.24; H, 6.24; N, 14.76.

5.2.21. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-5-yl]-ureido}-3-naphthalen-1-yl-propionylamino)-5-guanidino-pentanoic acid benzylamide (6). Starting from **24b**, compound **6** was obtained following the synthetic procedure reported for compound **5**: yield 0.75 g of crude product. MS/ESI (+), *m*/*z*: 875 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.56 (m, 6H), 1.80 (m, 2H), 2.30 (m, 4H), 2.40 (s, 3H), 2.67 (m, 2H), 3.37 (m, 1H), 3.52 (s, 2H), 3.62 (m, 1H), 4.46 (m, 3H), 4.95 (m, 1H), 5.31 (s, 2H), 6.80 (d, 1H), 7.19–7.77 (m, 17H). Anal. Calcd for $C_{48}H_{53}Cl_2N_9O_3$: C,65.89, H, 6.11, N, 14.41. Found: C, 65.78, H, 6.13, N, 14.44.

5.2.22. 2-(2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-5-yl]-ureido}-3-naphthalen-2-yl-propionylamino)-5-guanidino-pentanoic acid benzylamide (7). Starting from **24c**, compound **7** was obtained following the synthetic procedure reported for compound **5**: yield 0.72 g of crude product. MS/ESI (+), *m*/*z*: 875 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.50 (m, 6H), 1.75 (m, 2H), 2.35 (m, 4H), 2.49 (s, 3H), 2.70 (m, 2H), 3.04 (m, 1H), 3.29 (m, 1H), 3.55 (s, 2H), 4.42 (m, 3H), 4.89 (m, 1H), 5.30 (s, 2H), 6.82 (d, 1H), 7.06–7.60 (m, 17H). Anal. Calcd for $C_{48}H_{53}Cl_2N_9O_3$: C,65.89, H, 6.11, N, 14.41. Found: C, 65.93, H, 6.07, N, 14.45.

5.2.23. 2-[2-{3-[1-(2,6-Dichlorobenzyl)-2-methyl-3-pyrrolidin-1-ylmethyl-1*H*-indol-5-yl]-ureido}-3-(3,4-difluorophenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (8). Starting from **24d**, compound **8** was obtained following the synthetic procedure reported for compound **5**: yield 0.7 g of crude product. MS/ESI (+), *m*/*z*: 861 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.40 (m, 6H), 2.10 (m, 6H), 2.45 (s, 3H), 2.65 (m, 2H), 2.92 (m, 1H), 3.17 (m, 1H), 3.61 (s, 2H), 4.46 (m, 3H), 4.60 (m, 1H), 5.45 (s, 2H), 7.03–7.60 (m, 14H). Anal. Calcd for $C_{44}H_{49}Cl_2F_2N_9O_3$: C, 61.39; H, 5.74; N, 14.64. Found: C, 61.35; H, 5.79; N, 14.61.

5.3. Synthesis of 1,4-benzodiazepinic derivatives (compounds 9–16)

5.3.1. 7-Amino-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-2(3H)-one (27a). Nitrazepam **26a** (5 g, 0.018 mol), was dissolved in methanol (100 mL) and hydrogenated to the corresponding amino derivative **27a** using 10% Pd/C. The reaction mixture was stirred for 1 h at 2 atm of H_2, filtered, and then the solvent was removed in vacuo. The desired product **27a** was crystallized by diethyl ether/*n*-hexane: yield 4.1 g (91%). MS/ESI (+), *m*/*z*: 252 $[M+H]^+$.

5.3.2. 7-Amino-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one (27b). Clonazepam **26b** (3.5 g, 0.011 mol) was dissolved in methanol (100 mL) and acetic acid (10 mL) and hydrogenated to the corresponding amino derivative **27b** using 10% Pd/C. The reaction mixture was stirred at 3 atm until no more consumption of hydrogen was observed; then it was filtered and the solvent was removed in vacuo. The desired product **27b** was crystallized by diethyl ether/*n*-hexane: yield 2.95 g (94%). MS/ESI (+), *m*/*z*: 286/288 $[M+H]^+$.

5.3.3. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(4-methoxy-phenyl)-propionylamino]-5-[N^{ω} -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (28a). 7-Amino-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one (**27a**, 0.5 g, 2 mmol) was dissolved in DCM (50 mL) and the solution was cooled to −20 °C. *p*-Nitrophenylchloroformate (0.48 g, 2.4 mmol) and NMM (0.44 mL, 4 mmol) were added. After 30 min H-Tyr(Me)-Arg(Pbf)-NH-CH_2-C_6H_5 (1.4 g, 2 mmol) was added and the reaction mixture was allowed to warm to room temperature and stirred for an additional 16 h. Then the solution was ice-bath cooled and the solid **28a** was filtered and let air-dry furnishing 1.65 g of crude product that was used without further purification. MS/ESI (+), *m*/*z*: 971 $[M+H]^+$.

5.3.4. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4] diazepin-7-yl)ureido}-3-naphthalen-1-yl-propionylamino]-5-[N^{ω} -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (28b). Starting from **27a** and H-1-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **28b** was obtained following the synthetic procedure reported for compound **28a**: yield 1.7 g of crude product. MS/ESI (+), *m*/*z*: 991 $[M+H]^+$.

5.3.5. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4] diazepin-7- yl)ureido}-3-naphthalen-2-yl-propionylamino]-5-[N^{ω} -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (28c). Starting from **27a** and H-2-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **28c** was obtained following the synthetic procedure reported for compound **28a**: yield 1.75 g of crude product. MS/ESI (+), *m*/*z*: 991 $[M+H]^+$.

5.3.6. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(3,4-difluoro-phenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (28d). Starting from **27a** and H-3,4diF-Phe-Arg(Pbf)-NH-CH_2-C_6H_5, compound **28d** was obtained following the synthetic procedure reported for compound **28a**: yield 1.65 g of crude product. MS/ESI (+), *m*/*z*: 977 $[M+H]^+$.

5.3.7. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(4-methoxy-phenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (29a). Starting from **27b** and H-Tyr(Me)-Arg(Pbf)-NH-CH_2-C_6H_5, compound **29a** was obtained following the synthetic procedure reported for compound **28a**: yield 1.72 g of crude product. MS/ESI (+), *m*/*z*: 1006 $[M+H]^+$.

5.3.8. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chloro-phenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-naphthalen-1-yl-propionylamino]-5-[N^{ω} -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (29b). Starting from **27b** and H-1-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **29b** was obtained following the synthetic procedure reported for compound **28a**: yield 1.85 g of crude product. MS/ESI (+), *m*/*z*: 1026 $[M+H]^+$.

5.3.9. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-naphthalen-2-yl-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (29c). Starting from **27b** and H-2-Nal-Arg(Pbf)-NH-CH_2-C_6H_5, compound **29c** was obtained following the synthetic procedure reported for compound **28a**: yield 1.80 g of crude product. MS/ESI (+), *m*/*z*: 1026 $[M+H]^+$.

5.3.10. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(3,4-difluoro-phenyl)-propionylamino]-5-[N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)]-guanidino-pentanoic acid benzylamide (29d). Starting from **27b** and H-3,4diF-Phe-Arg(Pbf)-NH-CH_2-C_6H_5, compound **29d** was obtained following the synthetic procedure reported for compound **28a**: yield 1.78 g of crude product. MS/ESI (+), *m*/*z*: 1011 $[M+H]^+$.

5.3.11. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(4-methoxy-phenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (9). The intermediate 28a (1.65 g, 1.7 mmol) was dissolved in TFA (20 mL) and the mixture was stirred for 2 h at room temperature. The solution was evaporated to dryness in vacuo and the residue was triturated with diethyl ether giving 0.9 g of crude product 9, which was purified by RP-HPLC using the same condition reported for compound 1. MS/ESI (+), *m*/*z*: 718 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.41–1.75 (m, 4H), 2.78–2.81 (m, 1H), 2.92–2.99 (m, 1H), 3.01–3.06 (m, 2H), 3.66 (s, 3H), 4.20–4.38 (m, 5H), 4.41–4.49 (m, 1H), 6.73 (d, 2H), 7.20–7.74 (m, 8H), 7.95 (d, 2H), 8.10 (d, 2H), 8.27–8.41 (m, 3H). Anal. Calcd for $C_{39}H_{43}N_9O_5$: C, 65.26; H, 6.04; N, 17.56. Found: C, 65.21; H, 6.03; N, 17.58.

5.3.12. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*] [1,4]diazepin-7- yl)ureido}-3-naphthalen-1-yl-propionylamino]-5-guanidino-pentanoic acid benzylamide (10). Starting from **28b**, compound **10 was obtained following the synthetic procedure reported for compound 9: yield 1.1 g of crude product. MS/ESI (+), *m*/*z*: 738 $[M+H]^+$. ^1H NMR (DMSO-d_6)** δ 1.38–1.50 (m, 2H), 1.52–1.58 (m, 1H), 1.69–1.75 (m, 1H), 3.05–3.1 (m, 2H), 3.18–3.21 (m, 1H), 3.48–3.52 (m, 1H), 4.22–4.32 (m, 5H), 4.58–4.61 (m, 1H), 7.08 (d, 2H), 7.20–7.51 (m, 9H), 7.74 (d, 2H), 7.88 (d, 2H), 8.12 (d, 2H), 8.32–8.36 (t, 1H), 8.39 (d, 2H). Anal. Calcd for $C_{42}H_{43}N_9O_4$: C, 68.37; H, 5.87; N, 17.08. Found: C, 68.43; H, 5.88; N, 17.10.

5.3.13. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*] [1,4]diazepin-7- yl)ureido}-3-naphthalen-2-yl-propionylamino]-5-guanidino-pentanoic acid benzylamide (11). Starting from **28c**, compound **11** was obtained following the synthetic procedure reported for compound **9**: yield 1 g of crude product. MS/ESI (+), *m*/*z*: 738 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.36–1.47 (m, 2H), 1.49–1.55 (m, 1H), 1.71–1.78 (m, 1H), 3.00–3.12 (m, 2H), 3.21–3.25 (m, 1H), 3.46–3.54 (m, 1H), 4.20–4.41 (m, 5H), 4.48–4.55 (m, 1H), 7.12 (d, 2H), 7.25–7.62 (m, 9H), 7.77 (d, 2H), 7.91 (d, 2H), 8.05 (d, 2H), 8.28–8.32 (t, 1H), 8.35 (d, 2H). Anal. Calcd for $C_{42}H_{43}N_9O_4$: C, 68.37; H, 5.87; N, 17.08. Found: C, 68.41; H, 5.90; N, 17.12.

5.3.14. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(3,4-difluoro-phenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (12). Starting from **28d**, compound **12** was obtained following the synthetic procedure reported for compound **9**: yield 1 g of crude product. MS/ESI (+), *m*/*z*: 724 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.40–1.80 (m, 4H), 2.80–2.91 (m, 1H), 2.96–3.01 (m, 1H), 3.05–3.10 (m, 2H), 4.22–4.36 (m, 5H), 4.42–4.56 (m, 1H), 7.20–7.74 (m, 7H), 7.97 (d, 2H), 8.08 (d, 2H), 8.38 (d, 2H), 8.42–8.56 (m, 3H). Anal. Calcd for $C_{38}H_{39}F_2N_9O_4$: C, 63.06; H, 5.43; N, 17.42. Found: C, 63.10; H, 5.45; N, 17.38.

5.3.15. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(4-methoxy-phenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (13). Starting from **29a**, compound **13** was obtained following the synthetic procedure reported for compound **9**: yield 1.15 g of crude product. MS/ESI (+), *m*/*z*: 752 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.42–1.78 (m, 4H), 2.62–2.75 (m, 1H), 2.88–2.96 (m, 1H), 3.07–3.08 (d, 2H), 3.67 (s, 3H), 4.11 (s, 2H), 4.20–4.31 (m, 4H), 6.72 (d, 2H), 6.96 (s, 1H), 6.99–7.52 (m, 11H), 8.33 (d, 1H), 8.41 (t, 1H). Anal. Calcd for $C_{39}H_{42}ClN_9O_5$: C, 62.27; H, 5.63; N, 16.76. Found: C, 62.20; H, 5.65; N, 16.78.

5.3.16. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-naphthalen-1-yl-propionylamino]-5-guanidino-pentanoic acid benzylamide (14). Starting from **29b**, compound **14** was obtained following the synthetic procedure reported for compound **9**: yield 1.20 g of crude product. MS/ESI (+), *m*/*z*: 772 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.42–1.80 (m, 4H), 2.94–2.97 (m, 1H), 3.07–3.09 (d, 2H), 3.12–3.14 (m, 1H), 4.25–4.35 (m, 5H), 4.58–4.59 (m, 1H), 6.95 (s, 1H), 7.24–7.46 (m, 10H), 7.75 (d, 2H), 7.88 (d, 2H), 8.18 (d, 2H), 8.35 (t, 1H), 8.40 (d, 1H). Anal. Calcd for $C_{42}H_{42}ClN_9O_4$: C, 65.32; H, 5.48; N, 16.32. Found: C, 65.38; H, 5.53; N, 16.37.

5.3.17. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-naphthalen-2-yl-propionylamino]-5-guanidino-pentanoic acid benzylamide (15). Starting from **29c**, compound **15** was obtained following the synthetic procedure reported for compound **9**: yield 1.1 g of crude product. MS/ESI (+), *m*/*z*: 772 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.44–1.78 (m, 4H), 2.94–2.96 (m, 1H), 3.07–3.09 (d, 2H), 3.12–3.14 (m, 1H), 4.11 (s, 2H), 4.20–4.31 (m, 3H), 4.58–4.59 (m, 1H), 6.95 (s, 1H), 6.99 (d, 2H), 7.25–7.62 (m, 8H), 7.77 (d, 2H), 7.64 (s, 1H), 7.71 (d, 2H), 7.81 (t, 1H), 8.42 (m, 2H). Anal. Calcd for $C_{42}H_{42}ClN_9O_4$: C, 65.32; H, 5.48; N, 16.32. Found: C, 65.29; H, 5.49; N, 16.30.

5.3.18. 2-[2-{1-((Z)-2,3-dihydro-2-oxo-5-(2-chlorophenyl)-1*H*-benzo[*e*][1,4]diazepin-7-yl)ureido}-3-(3,4-difluoro-phenyl)-propionylamino]-5-guanidino-pentanoic acid benzylamide (16). Starting from **29d**, compound **16** was obtained following the synthetic procedure reported for compound **9**: yield 1.05 g of crude product. MS/ESI (+), *m*/*z*: 758 $[M+H]^+$. ^1H NMR (DMSO-d_6) δ 1.41–1.78 (m, 4H), 2.75–2.81 (m, 1H), 2.95–3.01 (m, 1H), 3.07–3.09 (d, 2H), 4.12 (s, 2H), 4.27–4.31 (m, 3H), 4.40–4.48 (m, 1H), 6.97 (s, 1H), 7.06–7.24 (m, 5H), 7.28 (d, 1H), 7.47 (m, 6H), 8.37 (d, 1H), 8.50 (t, 1H). Anal. Calcd for $C_{38}H_{38}ClF_2N_9O_4$: C, 60.19; H, 5.05; N, 16.63. Found: C, 60.23; H, 5.06; N, 16.65.

5.4. Pharmacological assay

5.4.1. Platelet aggregation assay.[13] Blood was collected from healthy human volunteers free of aspirin and other drugs for at least 8 days, into 3.8% trisodium citrate anticoagulant (1:10). The platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 180*g* for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation of the sample of PRP at 1000*g* for 20 min at room temperature. Before all platelet aggregation studies, the platelet count was determined on a Coulter Counter and adjusted to 300,000 platelet/μL with autologous PPP. Platelet aggregation was assayed by a Chronolog dual-channel aggregometer by recording the increase in light transmittance through a stirred suspension of PRP maintained at 37 °C in the cuvette. PRP was treated with variable concentrations of PAR-1AP to determine the threshold concentration for platelet aggregation. Platelet aggregation was measured in arbitrary unit as the initial rate of change in light transmittance in the first minute after the addition of PAR-1AP. The effect of each compound on platelet aggregation was assayed by adding the compounds at various concentrations to the cuvette, allowing the baseline to stabilize (10 min) and then adding threshold amounts of PAR-1AP. The aggregation was allowed to proceed for 5 min. The concentration of each compound to achieve full inhibition of platelet aggregation was determined. Platelet aggregation data were expressed as percentage of inhibition of the extent of chart deflection of compound-treated platelet sample compared with an untreated sample ×100.

5.4.2. Aorta ring assay.[14] Male Wistar rats (200–250 g, Nossan, Italy) were housed in an environment with controlled temperature (21–24 °C) and lighting (12:12 light–darkness cycle). Standard chow and drinking water were provided ad libitum. A period of 7 days was allowed for acclimatization of rats before undertaking any experimental manipulation. All the experiments were conducted following the principles of laboratory animal care (law No. 86/609/CEE), as well as specific national law (No. 116/1992). Animals were anaesthetized by inhalation of isoflurane and after exsanguinations the thoracic aorta was removed, cleaned of adherent connective tissue, and cut into rings 3 mm in length. The endothelium was removed by gently rubbing the intimate surface with moistened filter paper. Endothelium-denuded rings were mounted under 0.5 g of tension on 2.5 mL organ baths containing Krebs salt solution of the following composition (in mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$ 1.2; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $NaHCO_3$ 25.0; and glucose 11.7. The solution was maintained at 37 °C and bubbled with 95% O_2–5% CO_2 (pH 7.4). Developed tension was measured using an isometric force transducer (Basile; Italy) connected to a recorder. Rings were allowed to equilibrate for 60 min and the Krebs solution was replaced each 15 min. In each experiment aortic rings were firstly challenged with PE (10^{-6} M) until the responses were reproducible and then aortic rings were contracted with PAR-1AP. To assess the ability of the compounds to inhibit PAR-1AP-induced contraction aortic rings were incubated with each compound (15 min; 10 μM).

5.4.3. Statistical analysis. All results are reported as means ± SEM. To analyze the curve was used ANOVA followed by Bonferroni test as post tests. A value of $p < 0.01$ was taken as significant.

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