

Pharmacological evaluation of the novel thromboxane modulator BM-567 (I/II). Effects of BM-567 on platelet function

J.-M. Dogné^a, X. de Leval^a, P. Kolh^a, V. Sanna^a, S. Rolin^b, C. Michaux^c, M. Mauer^d, J.-L. David^e, B. Masereel^b, B. Pirotte^a

^a *Laboratory of Medicinal Chemistry, Department of Medicinal Chemistry, Natural and Synthetic Drugs Research Center, University of Liège, I, avenue de l'Hôpital, Tour 4 (+5), Bât B36, B-4000 Liège (Sart-Tilman), Belgium*

^b *Department of Pharmacy, University of Namur, B-5000 Namur, Belgium*

^c *Department of Molecular and Structural Chemistry, University of Namur, B-5000 Namur, Belgium*

^d *Department of Biostatistic, University Hospital, B-4000 Liège, Belgium*

^e *Department of Thrombosis and Hemostasis, University Hospital, B-4000 Liège, Belgium*

Abstract

The aim of this work was to evaluate the effects of BM-567 (*N*-pentyl-*N'*-[(2-cyclohexylamino-5-nitrobenzene)sulfonyl]urea), a torasemide derivative, on both thromboxane A₂ (TXA₂) receptors (TP) and thromboxane synthase of human platelets. The drug affinity for TP receptors of human washed platelets has been determined. In this test, BM-567 showed a high affinity (IC₅₀: 1.1±0.1 nM) for the TP receptors in comparison with BM-531 (IC₅₀: 7.8 ±0.7 nM) and sulotroban (IC₅₀: 931±85 nM), two TXA₂ antagonists. We also demonstrated that BM-567 prevented platelet aggregation induced by arachidonic acid (AA) (600 μM) (ED₁₀₀: 0.20 ± 0.10 μM), U-46619, a stable TXA₂ agonist (1 μM) (ED₅₀: 0.30 ± 0.04 μM) and collagen (1 μg ml⁻¹) (% of inhibition: 44.3 ± 4.3% at 10 μM) and inhibited the second wave of ADP (2 μM). Moreover, when BM-567 was incubated in whole blood from healthy donors, the closure time measured by the Platelet Function analyzer (PFA-100®) was significantly prolonged (closure time: 215 ± 21 s) by using collagen/epinephrine cartridges. Finally, at the concentration of 1 μM, BM-567 completely reduced the TXB₂ production from human platelets stimulated with AA (600 μM). These results indicate that BM-567 is a novel combined TXA₂ receptor antagonist and thromboxane synthase inhibitor characterized by a powerful antiplatelet potency.

1. INTRODUCTION

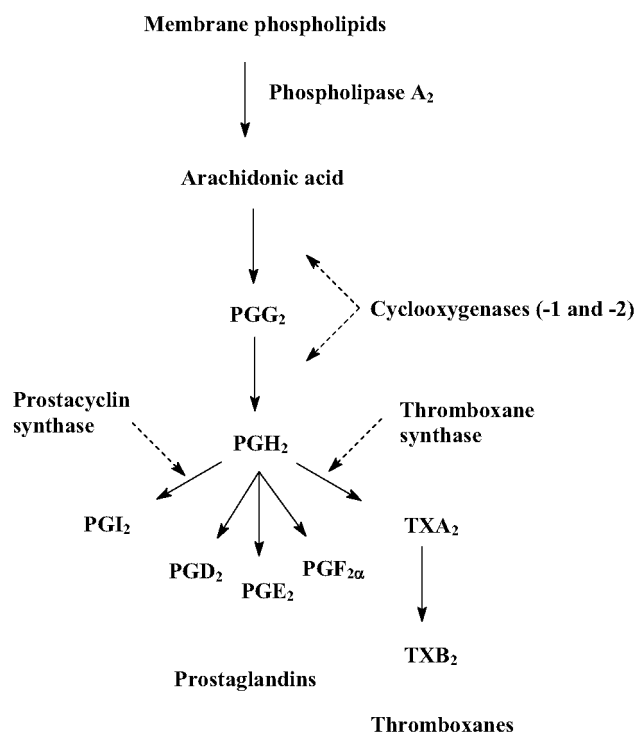
Platelet activation and vasoconstriction are intimately involved in thrombus formation. In particular, thromboxane A₂ (TXA₂), which causes platelet aggregation and vasoconstriction, plays an important role in the process of vascular thrombosis [1-6].

TXA₂ is a biologically potent arachidonate metabolite derived from the cyclo-oxygenase pathway [7]. Indeed, TXA₂ is formed by the action of thromboxane synthase on the prostaglandin endoperoxide H₂ (PGH₂) [8] mainly in activated platelets and macrophages [9,10]. Initially, there was a great interest in the potential use of TXA₂ receptor (TP) antagonists such as sulotroban. However, these compounds were too selective to completely inhibit platelet aggregation. In general, interest has switched to agents which are combined receptor antagonists and thromboxane synthase inhibitors such as ridogrel. The rationale behind this is that the TP receptor antagonist activity can block the aggregatory and vasoconstrictor actions of PGH₂ accumulating after inhibition of thromboxane synthesis and that this endoperoxide can be converted to either prostaglandin D₂ (PGD₂) by the platelets or prostacyclin (PGI₂) by the vessel wall, both of which increase platelet cyclic AMP levels to inhibit platelet activation [5] (Fig. 1).

According to the observation that the pyridinic sulfonylurea torasemide, a high ceiling loop diuretic, was able to induce a concentration-dependent relaxation of the canine coronary artery precontracted with the stable carboxylic TXA₂ [11], we designed and synthesized a series of its derivatives. Among these drugs, BM-500 (*N*-isopropyl-*N'*-[(2-metaboluyl-amino-5-nitrobenzene)sulfonyl]urea) emerged as one of the most potent TXA₂ receptor antagonists of this generation [12]. Later, we developed BM-531 (*N*-*tert*-butyl-*N'*-[(2-cyclohexylamino-5-nitrobenzene) sulfonyl]urea) which was demonstrated to be a stronger TXA₂ receptor

antagonist than BM-500 and to combine a thromboxane synthase inhibitory activity [13,14]. These encouraging results led us to design a new generation of derivatives chemically related to BM-531. Therefore, we selected BM-567 and examined its effects on TP receptors and thromboxane synthase of human platelets (Fig. 2). Its affinity for TXA_2 receptors has been determined and its antiaggregatory potency has been evaluated by its ability to prevent human platelet aggregation induced by arachidonic acid (AA), U-46619, a stable TXA_2 agonist, ADP and collagen. Moreover, we measured the activity of this drug on primary hemostasis measured by the Platelet Function Analyzer (PFA-100®). Finally, we examined the effects of BM-567 on TXB_2 production from platelets stimulated with AA in order to evaluate the thromboxane synthase inhibitory potency.

Fig. 1. Prostaglandins and thromboxane biosynthesis.

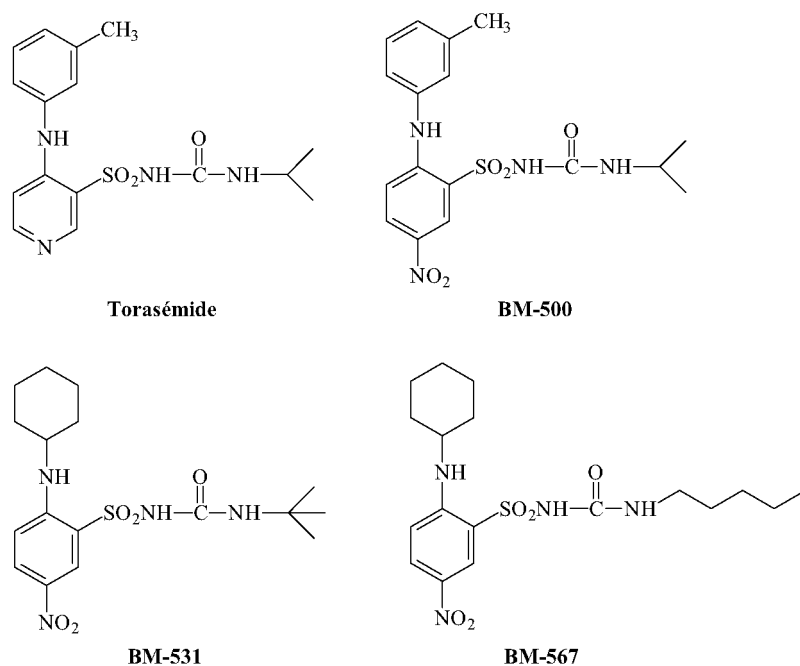


2. MATERIALS AND METHODS

2.1. Drugs and chemicals

Drugs were synthesized according to general synthetic pathways previously described [12,15]. Drug mother solutions were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%, a concentration which did not affect the parameters measured. Stock solutions of sodium arachidonate (5mM; Sigma, Belgium) were prepared in water. U-46619 (Cayman Chemical, Ann Arbor, MI) supplied in ethanolic solution was diluted with the incubation buffer. SQ-29548 was purchased from RBI (Bioblock, Illkirch, France) and sulotroban was synthesized according to a general procedure previously described [16]. The solution of collagen was provided by Horm (France) and [5,6-³H]SQ-29548 (46 Ci mmol⁻¹) was obtained from NEN Products (Brussels, Belgium).

Fig. 2. Chemical structures of torasemide, BM-500, BM-531 and BM-567.



2.2. Pharmacology

Platelet binding. The binding test realized on human washed platelets was performed according to a previously described method [13,14]. Human platelet-rich plasma (PRP) was provided by the Belgian Red Cross. Fractions (10 ml) of this plasma were centrifuged for 10 min at 1000 g (4°C). The supernatant was discarded, the pellet resuspended in NaCl (0.2%, 5 ml), mixed for 20 s and then diluted with NaCl (1.6%, 5 ml). The suspension was centrifuged again for 5 min at 1000 g (4°C). The supernatant was removed and the pellet was suspended in calcium- and magnesium-free Tyrode-Hepes buffer (mM: NaCl 137, KCl 2.7, NaH₂PO₄ 0.4, NaHCO₃ 12, D-glucose 5, Hepes q.s. ad pH 7.4) to a concentration of 2 x 10⁸ cells ml⁻¹. Freshly prepared samples of this suspension (500 µl) were incubated with [5,6-³H]SQ-29548 (5 nM final concentration, 100 µl) for 60 min at 25°C. The displacement was initiated by addition of the studied ligand dissolved in the same buffer (400 µl). After incubation (30 min, 25°C), ice-cold Tris-HCl buffer (10 mM, pH 7.4; 4 ml) was added and the sample was rapidly filtered through a glass-fiber filter (Whatman GF/C) and the tube was rinsed twice with ice-cold buffer (4 ml). The filters were then placed in plastic scintillation vials containing an emulsion-type scintillation mixture (4 ml) and the radioactivity was counted. The amount of [5,6-³H]SQ-29548 specifically bound to the human platelet TP receptors (B_s, %) was calculated:

$$B_s = 100 \times (B - \text{NSB}) / (B_t - \text{NSB}) \quad (1)$$

where B_t (total binding) and NSB (non-specific binding) are the radioactivity of [5,6-³H]SQ-29548 (5 nM) bound to the platelets incubated in the absence of any competing ligand and in the presence of unlabelled SQ-29548 (50 µM), respectively. B is the radioactivity of the filtered platelets incubated with [5,6-³H]SQ-29548 (5 nM) and the studied compound at a fixed concentration of 10⁻⁶M or at concentrations ranging from 10⁻⁵ to 10⁻¹⁰M. In each experiment, NSB varied between 5% and 7% of B_t. For the most potent drugs, three concentration-response curves were measured in triplicate using concentrations ranging from 10⁻⁵ to 10⁻¹⁰ M. The concentration of drug, which reduced the amount of specifically bound [5,6-³H]SQ-29548 by 50% (IC₅₀), was determined for each drug by non-linear regression analysis (GraphPad Prism software). The results are expressed as mean ± SDM.

Platelet aggregation. The antiplatelet potency was determined according to a previously described method [13]. Briefly, blood was collected by venipuncture from volunteers reported to be free from medication for at least 10 days and diluted (9:1) with trisodium citrate (3.8% w/w) in a polypropylene tube. The PRP was obtained from the supernatant fraction after centrifugation for 20 min at 90 g (25°C). The remaining blood was centrifuged at 2000 g for 5 min (25°C) and the supernatant gave the platelet-poor plasma (PPP). The platelet concentration of PRP was adjusted to 3 x 10⁸ cells ml⁻¹ by dilution with PPP. Aggregation tests were performed according to Born's turbidimetric method [19] by means of two-channel aggregometer (Chrono-log®). PPP was used to adjust

the photometric measurement to the minimum optical density. PRP (225 μl) was added in a silanized cuvette and stirred (1100 rev min^{-1}). Drug solution (20 μl) was then added and the mixture was incubated at 37°C for 3min. Platelet aggregation was initiated by addition of (5 μl) AA (600 μM final), U-46619 (1 μM final) ADP (2 μM final) and collagen (1 $\mu\text{g ml}^{-1}$ final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6min after addition of the inducer. The drug concentration preventing 100% of the platelet aggregation induced by AA (ED_{100}) was measured and expressed as mean \pm SDM ($n = 3$). The drug concentration reducing 50% of the platelet aggregation induced by U-46619 (ED_{50}) was calculated by non-linear regression analysis from at least three dose-response curves. The results are expressed as mean + SDM.

PFA-100. The PFA-100 system® (Dade Behring International®) has been described elsewhere in detail [17]. The system consists of an instrument and a disposable test cartridge where primary hemostasis is stimulated. Briefly, the system monitors platelet aggregation on a collagen-epinephrine-coated membrane as whole blood sample is aspirated under controlled flow conditions through a microscopic aperture cut into the membrane. The time required for the platelet plug to occlude the aperture is indicative of the platelet function in the sample. Blood samples collected by venipuncture from four healthy volunteers reported to be free from medication for at least 10 days and diluted (9:1) with trisodium citrate (3.8% w/w) in a polypropylene tube were incubated with or without the drug (10 μM) prior to test at room temperature typically for 5min with occasional mixing by gentle inversion. Then, the blood sample is added to the sample reservoir, and the test cartridge is added into the PFA-100® and incubated at 37°C. The sample is aspirated through a central aperture (150 μM diam.) cut into the membrane under steady vacuum created by displacement of the piston of a syringe pump connected to the vacuum chuck. Platelet activation and aggregation occurs at the area surrounding the aperture due to the presence of agonists and local shear conditions, eventually leading to complete occlusion of the aperture. The instrument monitors blood flow through the aperture and reports the time required for occlusion of the aperture by the platelet plug, thus defining the closure time (CT). The CT measured are expressed as mean + SDM ($n = 4$).

Thromboxane synthase activity. PRP preparation is identical to that described for the platelet aggregation experiments. Each drug was dissolved in dimethylsulfoxide (DMSO) and diluted with a Tyrode-Hepes buffer (mM: NaCl 137, KCl 2.7, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.4, D-glucose 5, NaHCO_3 12, Hepes; pH 7.4). To 900 ml of PRP, 50 ml NaCl, 0.9% and 10 ml of drug solution were added. After 6min incubation at 37°C under stirring (600 rpm), aggregation was induced by 40 ml sodium arachidonate (0.6 mM). After 4min, the reaction was stopped by adding 50 ml of indomethacin (0.02 M in ethanol). The sample was immediately centrifuged (17 500 g for 10 s) and the supernatant was removed and frozen (-78°C) until assayed for TXB_2 . Basal and maximal production of TXB_2 was estimated in the absence and in the presence of AA, respectively. Thromboxane synthase activity was expressed as the TXB_2 production, which was measured by using a competitive enzyme immunoassay (TXB_2 EIA Kit, Cayman Chemical Company).

3. RESULTS

In a first experimentation, we evaluated the affinity of BM-567 for human platelet TP receptors. Thereby, we measured the capacity of our drug to displace $[5,6\text{-}^3\text{H}]\text{SQ-29548}$, a potent competitive ligand of TP receptors, from its binding site on human washed platelets. We realized concentration-response curves and calculated IC_{50} values, which represent the drug concentration, needed to displace 50% of $[5,6\text{-}^3\text{H}]\text{SQ-29548}$ from TP receptors. Table 1 shows that BM-567 (IC_{50} : 1.1 ± 0.1 nM) is characterized by a higher affinity than sulotroban (IC_{50} : 931 ± 85 nM), BM-500 (IC_{50} : 79 ± 7.9 nM) and BM-531 (IC_{50} : 7.8 ± 0.7 nM).

In a second step, we determined the property of BM-567 to prevent human platelet aggregation induced by AA (600 μM), U-46619 (1 μM), ADP (2 μM) and collagen ($1\mu\text{gml}^{-1}$). When AA was used as inducer, BM-567 totally prevented the aggregation at the lower dose of 0.20 ± 0.10 μM (ED_{100}). Compared to the other TXA_2 receptor antagonists referenced in Table 1, BM-567 was much more active than both sulotroban (ED_{100} : 10.2 ± 2.1 μM) and BM-500 (ED_{100} : $14.0 \pm 2.3\mu\text{M}$) and no significant difference was found in comparison with BM-531 (ED_{100} : 0.125 ± 0.015 μM). When the TXA_2 stable agonist U-46619 was used as platelet aggregating agent, BM-567 (ED_{50} : 0.30 ± 0.04 μM) was slightly more active than BM-531 in terms of ED_{50} , which correspond to the concentration required preventing 50% of the aggregation. It was nevertheless much more potent than BM-500 and sulotroban (ED_{50} : 9.5 ± 2.4 and 11.3 ± 4.1 μM , respectively). The same ranking can be made when collagen was used as platelet aggregating agent. Indeed, at the concentration of 10 μM , BM-567 and BM-531, respectively, prevented $44.3 \pm 4.3\%$ and $42.9 \pm 4.9\%$ of platelet aggregation while BM-500 and sulotroban were not active. Finally, when ADP was used as inducer, none of the tested drugs prevented the initial phase of aggregation. On the other hand, BM-500 and sulotroban (10 μM) weakly prevented the second wave of platelet

aggregation while at the same concentration both BM-567 and BM-531 completely inhibited this secondary phase of ADP-induced aggregation. In this aggregometry study, torasemide (100 μ M) was inactive, irrespective of the inducer used (Table 1).

The antiplatelet potency of BM-567 has been confirmed on human whole blood by measuring with the platelet function analyzer (PFA-100®). In this study, we examined the effects of BM-567 on primary hemostasis. Compared to reference values (CT: 110 \pm 10 s), torasemide, BM-500 and sulotroban did not modify closure time of the central aperture of a membrane coated with a mixture of collagen and epinephrine (CT: 108 \pm 9; 114 \pm 8; 112 \pm 5 s, respectively) while both BM-531 and BM-567 significantly prolonged CT (CT: 175 \pm 24 and 215 \pm 21 s, respectively).

Finally, we examined the effects of BM-567 on the thromboxane synthase activity from human platelets. At the concentrations of 10 and 1 μ M, BM-567 totally prevented the production of TXB₂ by human platelets activated by AA (600 μ M). At the same dosage, the thromboxane synthase inhibitor furegrelate reduced the production of TXB₂ from 67.52% while torasemide and BM-500 showed no inhibitory potency. At lower concentration (0.1 μ M), BM-567 did not inhibit the production of TXB₂ anymore. In this test, BM-531 showed the same inhibitory profile as BM-567 (Fig. 3).

4. DISCUSSION AND CONCLUSIONS

In 1992, Uchida et al. [11] demonstrated that torasemide elicited a dose-dependent vasodilating action in the isolated canine coronary arteries contracted by the carbocyclic thromboxane A₂ (CTA₂), a stable analogue of the potent coronary vasoconstrictor TXA₂, whereas indapamide or furosemide had little effect on this preparation. Nevertheless, these effects were observed at non-therapeutic doses. In a previous work, we confirmed that torasemide possessed a weak affinity for TXA₂ receptor of human washed platelets and that this potency was not strong enough to prevent platelet aggregation induced by AA, U-46619, collagen and ADP, even at the dose of 100 μ M. Thereby, we synthesized and developed a series of its derivatives.

Table 1

Drugs	Binding ^a (IC ₅₀ , nM)	Aggregometry			PFA-100 ^e
		AA ^b (ED ₁₀₀ , μ M)	U-46619 ^c (ED ₅₀ , μ M)	Collagen ^d (%)	
Sulotroban	931 \pm 85	10.2 \pm 2.1	11.3 \pm 4.1	NI	112 \pm 5
Torasemide	2691 \pm 72	> 100	>100	NI	108 \pm 9
BM-500	79 \pm 7.9	14 \pm 2.3	9.5 \pm 2.4	NI	114 \pm 8
BM-531	7.8 \pm 0.7	0.125 \pm 0.015	0.48 \pm 0.09	42.9 \pm 4.9	175 \pm 24
BM-567	1.1 \pm 0.1	0.20 \pm 0.10	0.30 \pm 0.04	44.3 \pm 4.3	215 \pm 21

^a Drug affinity for TXA₂ receptor of human platelet: IC₅₀ represents the drug concentrations needed to displace 50% of [5,6-³H]SQ-29548 from TXA₂ receptor of human washed platelets.

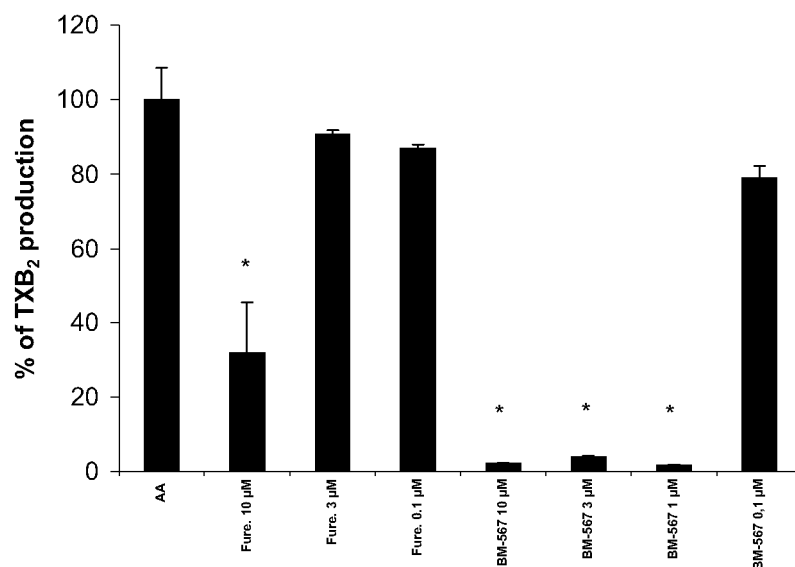
^b Drug concentrations required preventing 100% of the platelet aggregation induced by 600 μ M of AA.

^c Drug concentrations required preventing 50% of the platelet aggregation induced by 1 μ M of U-46619.

^d Inhibition percentage of platelet aggregation induced by collagen with a 10 μ M drug prevention (NI = no inhibition).

^e Closure times measured by PFA-100® (with drugs used at 10 μ M) compared to reference value (110 \pm 10 s). Results are expressed as mean \pm SDM (n \geq 3).

Fig. 3. Effects of furegrelate and BM-567 on thromboxane B₂ production induced by human platelets which expresses the thromboxane synthase inhibitory activity.



This work led to a first generation of TXA₂ receptor antagonist of which BM-500 was the most active. These encouraging results led us to design and synthesize a series of torasemide derivatives of second generation represented by BM-531, which was demonstrated as the first combined thromboxane receptor antagonist and thromboxane synthase inhibitor belonging to the sulfonyleurea class. Finally, with the aim of improving the pharmacological profile of BM-531, we designed different other derivatives of which BM-567 emerged. Indeed, we demonstrated that BM-567 strongly displaced [5,6-³H]SQ-29548 from TXA₂ receptor of human washed platelets with a higher potency than BM-500, BM-531 and sulotroban. We also confirmed its powerful TXA₂ antagonism and antiplatelet potency using standard aggregometric techniques and the recently developed platelet function analyzer (PFA-100®). In these tests, BM-567 was at least as active as BM-531, while BM-500 and sulotroban were less potent. Finally, we confirmed that BM-567 also reduced the production of TXB₂ by human platelets. This effect cannot be attributed to a cyclo-oxygenase inhibition since we demonstrated that BM-567 did not prevent the prostaglandin E₂ (PGE₂) production induced by both ovine COX-1 and COX-2 separately incubated with AA. This result confirms the thromboxane synthase inhibitory activity of BM-567. This has the advantage that other AA metabolites can still be produced. Thus, in the setting of platelet activation, as would occur locally at a vascular injury site, the platelet-produced endoperoxides can be taken up by other cells (smooth muscle cells, endothelial cells and white blood cells) which can then produce PGI₂ and other antithrombotic prostanoids [18,19]. Therefore, the antithrombotic effect due to the presence of locally produced prostacyclin could exceed that expected by only blocking TP receptors. Additionally, the conversion of the endoperoxides to E-type prostaglandins could help to reduce thrombus formation due to their vasodilatory action. A variety of compounds with both activities have been reported. Ridogrel was the first putative dual inhibitor of TXA₂ to be tested clinically, but it yielded disappointing clinical results because its receptor affinity (IC₅₀: 1.7 µM) was demonstrated too weak in regards to its thromboxane inhibitory (IC₅₀: 0.004 µM) effects to adequately qualify as a compound with a dual mode of action [20]. This is not the case with BM-567 which exhibits a combined activity on both TP receptors and thromboxane synthase at a similar concentration range. BM-567 was found slightly more active than BM-531 in preventing platelet aggregation induced by U-46619 and prolonging closure time with collagen-epinephrine cartridges from PFA-100®. From a structural point of view, both drugs feature an acidic sulfonyleurea moiety which appears to mimic the carboxylic acid function found in all prostanoids and the majority of their pharmacological modulators [21]. In conclusion, this work led to the discovery of BM-567, an original, well-balanced non-carboxylic TXA₂ receptor antagonist and thromboxane synthase inhibitor. This compound has been selected for further *in vivo* experiments as antithrombotic agent.

Acknowledgements

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