JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.063610

Pharmacological characterization of BM-573, a novel thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor in a rat model of arterial thrombosis and its effects on bleeding time

Jean-Michel Dogné, Julien Hanson, Xavier de Leval, Philippe Kolh, Vincent Tchana-Sato, Laurence de Leval, Stéphanie Rolin, Alexandre Ghuysen, Patrick Segers, Bernard Lambermont, Bernard Masereel and Bernard Pirotte

Natural and Synthetic Drug Research Center, University of Liège, Liège, Belgium (JMD, JH, XdL, BP); Experimental Hemodynamics Laboratory (HemoLiège), University Hospital of Liège, Liège, Belgium (PK, VT, AG, BL); Department of Pathology, University Hospital of Liège, Liège, Belgium (LdL); Department of Pharmacy, University of Namur, Namur, Belgium (SR, BM); Hydraulics Laboratory, Institute Biomedical Technology, Ghent University, Belgium (PS).

a) Running title:

BM-573, a novel antithrombotic agent

b) Corresponding author:

Dr. Jean-Michel Dogné

Natural and Synthetic Drug Research Center, University of Liège, Avenue de l'Hôpital, 1, B36, B-4000 Liège, Belgium.

E-mail : Jean-Michel.Dogne@ulg.ac.be

Phone: 003243664382 ; Fax: 003243664362

c)

Number of text pages: 23, including this page and references

Number of tables: 0

Number of figures: 9

Number of references: 37

Number of words in the abstract: 244

Number of words in the introduction: 561

Number of words in the discussion: 1373

d) List of nonstandard abbreviations:

thromboxane A_2 (TXA_2); prostaglandin endoperoxide H_2 (PGH_2); aspirin (ASA); thromboxane receptor (TP); prostacyclin (PGI_2); platelet rich plasma (PRP); platelet poor plasma (PPP), time to occlusion (TTO); bleeding time (BT); thromboxane receptor antagonist (TXRA); thromboxane synthase inhibitor (TXSI).

e) Recommended section:

Cardiovascular (& Thrombosis)

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.063610
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The present study was undertaken in order to characterize the antiplatelet and antithrombotic effects of BM-573 (*N*-*tert*-butyl-*N*'-[2-(4'-methylphenylamino)-5-nitrobenzenesulfonyl]urea), an original combined thromboxane receptor antagonist and thromboxane synthase inhibitor in rats, and to determine its effects on mice bleeding time. Intraperitoneal injection of a single dose of 5 mg/kg of BM-573 to rats inhibited U-46619-induced washed platelet aggregation 30 minutes, 1, 2 and 4 hours after drug administration with a maximum antiplatelet effect observed after 1 and 2 hours. In a rat model of thrombosis induced by ferric chloride application on the abdominal aorta, BM-573 at doses of 5, 2, 0.5 and 0.2 mg/kg significantly reduced the thrombus weight by 92.53 %. 80.20 %, 64.75 % and 18.21 %, respectively. Time to occlusion of abdominal aorta in BM-573-treated group (41.50+/-5.21 min) was significantly prolonged compared to the vehicle-treated rats (16.16+/-0.79 min). BM-573, as furegrelate, seratrodast and acetylsalicylic acid did not affect the tail bleeding time induced by tail transection in mice compared to vehicle-treated mice. Moreover, BM-573, a close derivative of the loop diuretic torasemide failed to induce a significant increase in diuresis in rat and did not produce a decrease in blood glucose concentration as observed with the sulfonylurea glibenclamide. In conclusion, we have demonstrated that the nitrobenzenic sulfonylurea BM-573, an original combined thromboxane receptor antagonist and thromboxane synthase inhibitor, is a potent antithrombotic agent that does not affect bleeding time. Moreover, BM-573 lost the diuretic property of torasemide and has no impact on glycemia.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.063610
This article has not been copyedited and formatted. The final version may differ from this version.

The isozymes cyclooxygenase (COX)-1 and -2 catalyze the conversion of arachidonic acid into thromboxane A_2 (TXA_2) and prostaglandins (PGs). The eicosanoid TXA_2 is the major COX-1 product of arachidonic acid metabolism in platelets. It is formed by the action of thromboxane synthase on the prostaglandin endoperoxide H_2 (PGH_2). TXA_2 causes vasoconstriction, bronchoconstriction and irreversible platelet aggregation (Hamberg et al. 1975; Svensson et al. 1976; Moncada et al. 1978; Bhagwat et al. 1985; Fiddler & Lumley 1990). Furthermore, recent studies have pointed out a significant stimulatory role of TXA_2 in the proliferation of vascular smooth muscle cells and mitogenesis (Pakala & Benedict 1998; Koba et al. 2000). Its biosynthesis is increased in syndromes of platelet activation, such as myocardial infarction (Eikelboom et al. 2002), unstable angina (Hamm et al. 1987), thrombosis and thrombotic disorders (Saldeen et al. 1993) but also in other pathophysiological states such as pulmonary hypertension, asthma and septic shock (Dogné et al. 2003). Inhibition of thromboxane synthesis underlies the efficacy of aspirin (ASA) in significantly reducing the incidence of cardiovascular death, myocardial infarction, and stroke in high-risk patients (Collins et al. 1994). Initially, there was a great interest in the potential use of TXA_2 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 and terbogrel. The rationale behind this is that the TP receptor antagonist activity can block the aggregatory and vasoconstrictor actions of both TXA_2 and PGH_2. Moreover, this endoperoxide accumulating after inhibition of thromboxane synthesis can be converted to either prostaglandin D_2 (PGD_2) by the platelets or prostacyclin (PGI_2) by the vessel wall, both

of which increase platelet cyclic AMP levels and inhibit platelet activation (Cheng et al. 2002, de Leval et al. 2003, Dogné et al. 2003) (Figure 1).

BM-573 is a molecule derived from the pyridinic sulfonylurea torasemide, a loop diuretic. It is obtained by the replacement of the pyridine ring of torasemide with nitrobenzene and the presence of a *tert*-butyl group on the distal nitrogen atom of the sulfonylurea moiety (Figure 2). These modifications improved the TXA_2 antagonism and revealed TXA_2 synthase inhibitory potency. Indeed, we demonstrated that this original molecule showed a high affinity (IC_{50}: 1.3 nM) for the TP receptors of human platelets. Moreover, BM-573 was found to be a potent inhibitor of human platelet aggregation induced by arachidonic acid (ED_{100}: 0.13 µM) or by the stable TXA_2 mimetic U-46619 (ED_{50}: 0.24 µM). BM-573 also relaxed the isolated rat thoracic aorta (ED_{50}: 28.4 nM) contracted by U-46619 and completely reduced the platelet production of the thromboxane B_2, the stable TXA_2 metabolite in blood, induced by arachidonic acid (Rolin et al. 2001).

These promising *in vitro* pharmacological properties led us to study the pharmacological profile of BM-573 *in vivo* as antiplatelet and antithrombotic agent. Thus, we measured the blood concentration of BM-573 following intraperitoneal administration in rats at different times, and evaluated the effects on *ex vivo* platelet aggregation. We determined the antithrombotic activity of our drug in a ferric chloride abdominal arterial thrombosis model in rat and evaluated its effects on bleeding time (BT) in mice. Finally, we studied the effects of the intraperitoneal injection of BM-573 in rat on diuresis and glycaemia and compared them to those induced by torasemide and the sulfonylurea glibenclamide, respectively.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.063610
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Drugs

BM-573 (*N*-*tert*-butyl-*N*'-[2-(4'-methylphenylamino)-5-nitrobenzenesulfonyl]urea) and torasemide (*N*-propyl-*N*'-[(4-m-toluidino-3-pyridyl)sulfonyl]urea) were synthesized in our laboratory. The sodium salts were dissolved in propyleneglycol and diluted with physiological saline. Furegrelate (5-(3-pyridinylmethyl)-2-benzofurancarboxylate) and U-46619 (9.11-dideoxy-9.11-methanoepoxy-prostaglandin F_2) was purchased from Cayman Chemical (MI, USA). They were dissolved in ethanol and diluted with physiological saline. Acetylsalicylic acid and glibenclamide were purchased from Sigma-Aldrich (Belgium). Seratrodast was isolated from Bronica^{TM}, a generous gift from Takeda Chemical Industries (Osaka, Japan) The sodium salts were dissolved in propyleneglycol and diluted with physiological saline.

Animals

Male Sprague–Dawley rats, weighing 250–300 g and 8- to 12-week-old male and female mice were housed in a temperature-controlled room before being used in the present experiments. All experimental procedures and protocols used in this investigation have been carried out in accordance with the Declaration of Helsinki (Publication No. 85-23. revised 1985) and were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Liege.

Ex vivo platelet aggregation in rat and BM-573 measurements

Male Sprague-Dawley rats were anesthetized with pentobarbital sodium (50 mg/kg, IP). Blood (2.5 ml) was drawn from the abdominal aorta into a tube containing 0.3 ml of trisodium citrate (3.2% w/v) as an anticoagulant. Platelet-rich plasma (PRP) was prepared by

centrifugation at 180 g for 10 minutes (15°C). Platelet-poor plasma (PPP) was obtained by centrifugation of remaining blood at 2205 g for 10 minutes (15°C). The platelet-aggregation method was modified from Yokoyama et al. (1994). In brief, PRP was centrifuged at 1000 g for 20 minutes, and the supernatant was discarded. The platelets were suspended in washing buffer at a volume equal to the original plasma volume and centrifuged at 1000 g for 20 minutes. The platelets were re-suspended in a suspension buffer and adjusted to a concentration of $5x10^8$ cells/ml.

The washing buffer contained 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 12mM $NaHCO_3$, 0.4mM NaH_2PO_4, 0.05 trisodium citrate, and 0.1% glucose (w/v). The pH was adjusted to 6.5 with HCl. The suspension buffer contained 113mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 24mM $NaHCO_3$, 10mM HEPES-NaOH (pH 7.4) and 0.1% glucose (w/v) (Li et al. 1998).

Platelet aggregation was studied using the turbidimetric method of Born (Born & Cross 1963) in an aggregometer Chronolog Corporation™, as previously described (Loï et al. 1998). A volume of 240 µl of the platelet suspension was placed in a glass turbidity tube and warmed at 37°C for 3 minutes in the cells of the aggregometer. Three minutes later, 2 µl of calcium chloride solution was added to obtain a final concentration of calcium equal to 0.2 mM. One minute later, 10 µl of U-46619 (final concentration of 1 µM) was added. Changes in light transmission were recorded 10 minutes after stimulation with U-46619. The extent of aggregation was estimated by the percent of maximum increase in light transmission, with the buffer representing 100% transmittance.

To examine the duration of the inhibitory effects of the BM-573 on platelet aggregation, the drug was administered intraperitoneally in rats 30 minutes, 1, 2, 4, 7 and 10 hours prior blood (2.5 ml) was drawn from the abdominal aorta. BM-573 measurements were performed on blood samples at the same times by using a high-performance liquid chromatography (HPLC) technique.

Ferric chloride-induced rat arterial thrombosis

Thrombus weight and histopathology

The experiments were carried out according to the modification of the method described by Kurz et al. (1990). Rats were anesthetized with pentobarbital sodium (50 mg/kg, IP). After an abdominal midline incision, the abdominal aorta was exposed carefully. A filter paper disk (diameter 8 mm) saturated with 50% (w/v) ferric chloride solution was placed on the surface of the artery for 10 min. The artery was isolated 10 min after removing the disk and then the rat was euthanized. The removal abdominal artery was opened lengthwise and the thrombus scraped out and placed on filter paper to remove any water. Its wet weight was measured immediately. Results are expressed in mg of thrombus weight by kg of rat weight. BM-573 (5, 2, 0.5, 0.2 mg/kg), placebo, torasemide (5 mg/kg), furegrelate (5 mg/kg) were injected intraperitoneally 1 and 2 hours prior to application of ferric chloride.

Cross-sections of abdominal rat aorta treated with the paper filter soaked with a solution of ferric chloride as described above were fixed overnight in buffered formalin and embedded in paraffin. Four-micron sections were cut and stained with haematoxylin and eosin.

Rat abdominal aortic blood flow measurements

A filter paper disk (diameter 8 mm) saturated with 50% (w/v) ferric chloride solution was placed on the surface of the rat artery for 10 min following the same procedure as described in II.4.1. The rat abdominal aortic blood flow expressed in ml/min was recorded continuously by an ultrasonic Doppler flow probe (Transonic systems, Ithaca, NY, USA). The time to the occlusion (TTO) of the abdominal aorta was measured in minutes.

Bleeding time in mice

Bleeding times were assessed according to a previously reported method (Ma et al. 2001). In brief, mice were placed in a holder, and their tails were transacted with a surgical blade 1 cm proximal from the tip. The remaining tail was immersed immediately into physiological saline maintained at 37°C, and the time during which visible bleeding was observed was measured. BM-573, furegrelate, seratrodast, aspirin, ticlopidine and vehicle were given intraperitoneally 1 hour prior to tail cutting.

Diuresis

Experiments were conducted as described previously (Masereel et al. 1993). Rats (250–300 g) received an intraperitoneal injection of BM-573 or torasemide at the dose of 5 mg/kg. Rats were allowed free access to food and water until the beginning of the experiment and were housed in groups of three in metabolism cages. Urine was collected during 4 h after drug administration and diuresis (ml/kg) was expressed as mean of urinary volume (ml) collected from the cage.

Glycemia

The methodology was performed according to Lebrun et al. (2000). In brief, rats were allowed to settle in for at least 3 days in the laboratory before use. They had free access to water and received a standard pellet diet. Conscious rats were placed for 60 min prior blood sampling and throughout the duration of the study in a small cabinet. At zero time, BM-573 or glibenclamide were administered intraperitoneally at a dosage of 20 mg/kg. Control animals received an equivalent volume of water, propyleneglycol (50% total volume); 1.5 ml/kg body weight. Blood was taken from the tail at time 5, 30, 60, 120 and 180 min. Blood glucose was

measured, in duplicate, using a reagent strip in combination with a glucometer (GLUCO TOUCH, LifeScan, Johnson & Johnson company, Belgium).

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) and statistical significance was determined by Student's t-test and the chi-square test. Probability values of less than 0.05 were considered to be significant.

Results

Ex vivo platelet aggregation in rat and BM-573 blood measurements

Intraperitoneal injection of a single dose of 5 mg/kg of BM-573 to rats inhibited U-46619-induced washed platelet aggregation 30 minutes, 1, 2 and 4 hours after drug administration. The maximum antiplatelet effect was observed 1 and 2 hours after BM-573 administration (Figure 3). Blood levels of BM-573 measured by HPLC were maximum 1 hour after BM-573 injection and decreased time-dependently after this period (Figure 4).

Antithrombotic effect of BM-573

Thrombus weight

Application of ferric chloride (50% w/v) for 10 minutes to the abdominal aorta induced marked thrombi in vehicle-treated rats (8.3973 +/- 0.369 mg/kg). In this group, the mixed thrombi composed of both fibrin and platelets, were adherent to the vessel wall at the site of ferric chloride contact as revealed by light micrographs of abdominal rat aorta sections stained with hematoxylin–eosin (Figure 5).

The intraperitoneal injection of BM-573 at doses of 5, 2, 0.5 and 0.2 mg/kg one hour before ferric chloride application resulted in a significant reduction of thrombus weight by 92.53 %. 80.20 %, 64.75 % and 18.21 %, respectively. When BM-573 was administered intraperitoneally two hours before ferric chloride treatment at doses of 5 and 2 mg/kg, the thrombus weight was reduced by 61.34 % and 44.58 %, respectively. No significant effects were observed with torasemide, furegrelate or seratrodast on thrombus formation when administered intraperitoneally at 5 mg/kg one or two hours before thrombus induction (Figure 6).

Time to occlusion

Time to occlusion (TTO) of abdominal aorta in vehicle-treated rats ranged from 13 min to 18 min after filter paper soaked in 50% solution of $FeCl_3$ application with an average TTO of 16.16+/-0.79 min. After intraperitoneal injection of 5 mg/kg BM-573 one hour before ferric chloride application, the average TTO measured was 41.50+/-5.21 min. In the same conditions, torasemide, seratrodast and furegrelate did not prolong TTO.

Effect of BM-573 on bleeding time in mice

BM-573, furegrelate, seratrodast and acetylsalicylic acid (5 mg/kg, IP, 1h pre-treatment) did not affect the tail BT induced by tail transection in mice compared to vehicle-treated mice. Aspirin tented to prolong the tail bleeding time although its effect failed to reach significance. In the same conditions, ticlopidine significantly prolonged BT up to 247s (Figure 7).

Diuretic properties of BM-573 in rat

Diuretic properties of BM-573 and torasemide were studied time-dependently after intraperitoneal administration of 5 mg/kg in rat. Figure 8 shows that the single intraperitoneal administration of torasemide induced a significant increase in diuresis in rat. Cumulated urine volumes measured in the BM-573-treated group do not differ from those in the vehicle-treated group.

Effects of BM-573 on blood glucose concentration

In control rats receiving the vehicle, the blood glucose level was stable during 180 min. The glycaemia averaged 115.2 ± 9.7 mg/100 ml at time –5 min and 109.4 ± 16.6 mg/100 ml at the 180^{th} min. The intraperitoneal injection of BM-573 did not provoke a decrease in glycaemia:

at the –5 min. the blood glucose level amounted to 116.6 ± 12.3 mg/100 ml and 105.2 ± 11.9 mg/100 ml at 180 min. The hypoglycaemic effect of glibenclamide observed was rapid and marked. On the 60^{th} min, the glycaemia averaged 67.4 ± 7.8 mg/100 ml after the administration of 20 mg/kg (Figure 9).

Discussion

Thromboxane A_2 is both a powerful aggregating mediator and a constrictor of blood vessels. TXA_2 levels are elevated in conditions associated with platelet activation leading to thrombosis, including unstable angina, myocardial infarction and cerebral ischemia. Aspirin is the most common drug used to prevent TXA_2 production. Indeed, low doses of aspirin selectively inhibit the formation of TXA_2 via cyclooxygenase-1 inhibition without altering the basal biosynthesis of cardioprotective prostacyclin produced via cyclooxygenase-2 by endothelial cells. Furthermore, ASA causes complete enzyme inhibition, without the recovery of enzyme activity. The effect of ASA in the prevention of ischemic events has been well documented in many older and recent clinical trials. It is clear from these studies that ASA, alone or in combination with other antithrombotics, significantly reduces the incidence of cardiovascular death, stroke, and myocardial infarction (Mangano et al. 2002; Gorelick PB et al., 2003). Thus, acetylsalicylic acid has been a drug of choice as antiplatelet agent. However, aspirin sensitivity has been noted, leading to asthma and Reye's syndrome (Pinsky et al. 1988). Moreover, ASA use is associated with gastroduodenal mucosal damage and increased risk of upper gastrointestinal bleeding (Awtry & Loscalzo 2000). Since inhibition of thromboxane synthesis underlies the efficacy of aspirin, specific thromboxane receptor antagonists (TXRAs) such as seratrodast and thromboxane synthase inhibitors (TXSIs) such as ozagrel have been developed. Both thromboxane receptor antagonists and thromboxane synthase inhibitors show interesting pharmacological characteristics. TXSIs can result in an

increase of the synthesis of the antiaggregatory and vasodilatory prostacyclin and TXRAs can block the action of both TXA_2 and PGH_2 at a receptor level. Thereby, a solution to optimize the therapeutic benefit of both types of agents used alone was to combine their properties in the same molecule. Indeed, to support this concept, a series of double-blind, placebo-controlled, crossover studies in healthy volunteers were carried out using a co-administration of the TXRA sulotroban and the TXSI dazoxiben. Results obtained confirmed the greater interest for co-administration of both thromboxane modulators than their independent use (Dogné et al. 2000, 2003).

In previous *in vitro* and *ex vivo* studies, it has been demonstrated that BM-573 was a potent combined compound able to reduce TXA_2 production by thromboxane inhibition and to prevent the action of TXA_2 (or PGH_2) by blocking the TXA_2 receptors. The present study demonstrated that this dual TXA_2 receptor antagonist and TS inhibitor prevent platelet aggregation and thrombus formation without affecting bleeding time. Indeed, intraperitoneal injection of BM-573 (5 mg/kg) to rats inhibited U-46619-induced washed platelet aggregation. The maximum antiplatelet effect was observed 1 and 2 hours following BM-573 corresponding to the higher blood levels of BM-573 assayed by HPLC. This result indicates that BM-573 is active as TXRA *in vivo* and is rapidly metabolised after IP injection. Indeed, BM-573 blood levels decrease significantly 2 hours following the injection and the *ex vivo* antiplatelet effect cannot be observed after 7 hours. In support to these pharmacokinetic data, we evaluated the antithrombotic effect of BM-573 on ferric chloride-induced arterial thrombosis in rats. We chose this model because the development of thrombi in response to ferric chloride-induced vascular injury was described to be physiologically relevant (Kurz et al. 1990). We observed, In this model, that application of ferric chloride to the abdominal aorta induced marked mixed thrombi composed of activated platelets, fibrin strands and entrapped erythrocytes. These thrombi were adherent to the vessel wall at the site of ferric

chloride contact as revealed by light micrographs of abdominal rat aorta sections (Figure 5). By using a ferric-chloride solution concentration of 50% w/v, the time to form an occlusive thrombus that induced the occlusion of rat abdominal aorta was less than 20 minutes in all experiments with an average of 16.16 min. This is in accordance with data of Tanaka and collaborators who performed a similar thrombus model in rats (Tanaka et al. 2000). In this study, BM-573 clearly prevented thrombus formation following ferric chloride-induced vascular injury with a maximum activity observed when injected 1 hour before thrombus formation. Both the histopathological examination and time to occlusion measurements confirmed this antithrombotic effect. Moreover, the TXRA seratrodast and TXSI furegrelate were inactive. This result confirms our previous data supporting that seratrodast, an antiasthmatic agent, was a weak human platelet thromboxane receptor antagonist (Dogné et al. 2003). Furegrelate is a weak TXSI that only prevent human platelet aggregation induced by arachidonic acid or U-46619 at high concentrations. These findings suggest that potent thromboxane modulators such as BM-573 are effective in preventing acute and mixed-type arterial thrombosis. Our results are supported by two previous reports indicating that, in a similar rat model of ferrous chloride-induced artery thrombosis, two selective TP-receptor antagonists, Z-335, given orally, and BMS-180291, given intravenously, decreased the weight of arterial thrombi (Tanaka et al., 2000). We thereafter examined the effects of BM-573 at the antithrombotic dose of 5 mg/kg on bleeding time in mice compared to seratrodast, furegrelate, aspirin and ticlopidine. The bleeding time measured in the vehicle-group (129.3+/-15.4s) was coherent with results obtained by Ma et al. (2001). In this test, all thromboxane modulators failed to prolong bleeding time. Aspirin tented to prolong the tail bleeding time although its effect was not statistically significant. These results confirms that TXA_2/PGI_2 balance is not a major factor regulating bleeding time in acute models (Tanaka et al. 1998). The causes of the separation of antithrombotic and bleeding time effects of BM-573 remain still unknown. In

contrast, ticlopidine, an ADP receptor antagonist significantly increased bleeding time tendency as observed by different authors (Kim et al. 1998; Foster et al. 2001). Finally, we examined both the diuretic and hypoglycaemic properties of BM-573 compared to torasemide and glibenclamide, respectively. Indeed, BM-573 was originally designed from a pharmacomodulation study of the loop diuretic torasemide that was found to be a weak thromboxane receptor antagonist at non therapeutic dosage (Uchida et al. 1992). Thus, the diuretic effects of torasemide and BM-573 were compared in rats over 10 hours after intraperitoneal injection of a single dose of 5 mg/kg. As expected, torasemide produced a significant increase in urinary volume over the 10-hour period while diuresis induced by BM-573 was not different from the vehicle-treated group. From a pharmacological point of view, torasemide is a diuretic that acts by inhibiting $Na^+K^+2Cl^-$ co-transport in the thick ascending limb of the loop of Henle. Thus, it can be postulated that BM-573 lost the diuretic property of torasemide because of a lack of affinity for the $Na^+K^+2Cl^-$ co-transport in the thick ascending limb of the loop of Henle (Wittner et al. 1986). This hypothesis can be explained on the basis of the structure-activity studies realized on torasemide derivatives. Indeed, torasemide is a pyridine-3-sulfonylurea derivative. Several studies demonstrated that the sulfonylurea moiety can be substituted by a sulfonylthiourea or a sulfonylcyanoguanidine without dramatically affecting the diuretic properties of torasemide (Masereel et al. 1993). Nevertheless, Wittner and collaborators demonstrated that if the pyridine ring is replaced by a NO_2-substituted phenyl ring such as with BM-573, the inhibitory potency for the $Na^+K^+2Cl^-$ co-transport system of the cortical thick ascending limb was lost (Wittner et al. 1987). *In vivo* experiments performed by our group with other nitrobenzenic sulfonylureas derivatives also confirmed the loss of the diuretic property. The oral hypoglycaemic glibenclamide, a specific blocker of the ATP-sensitive K+ channel, is the other sulfonylurea derivative that emerged as original TXRA. Indeed, in isolated ring segments of dog coronary artery, glibenclamide has been

shown to cause a reduction of both spontaneous isometric force and contractions induced by U-46619 (Cocks et al. 1990; Stanke et al. 1998). Thus, the aim of the last experiment was to evaluate the hypoglycaemic property of the sulfonylurea BM-573 compared to glibenclamide in rats. In this test, while the hypoglycaemic effect of glibenclamide injected in rats was rapid and marked as observed by Lebrun et al., BM-573 failed to produce a decrease in blood glucose concentration.

In conclusion, we have demonstrated that the nitrobenzenic sulfonylurea BM-573 is a potent antithrombotic agent that does not affect bleeding time. These effects observed *in vivo* can be explained by the pharmacological properties of BM-573 at a cellular level. It was indeed characterized as an original combined thromboxane receptor antagonist and thromboxane synthase inhibitor. Moreover, BM-573 lost the diuretic property of torasemide and has no impact on glycemia. The chemical structure of BM-573 offers a new template for the design of original and potent TXA_2 modulators useful in thrombotic disorders.

Acknowledgements

The authors want to thank Patricia Benoit for advice on these experiments and Philippe Neven for excellent technical assistance.

References

Awtry EH, Loscalzo J (2000). Aspirin. *Circulation* **101**:1206-1218.

Bhagwat SS, Hamann PR, Still WC, Bunting S, Fitzpatrick FA. Synthesis and structure of the platelet aggregation factor thromboxane A_2 (1985). *Nature* **315**:511-513.

Born GV, Cross MF. The aggegation of blood platelets (1963). *J Physiol* **168**:178-195.

Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA (2002). Role of prostacyclin in the cardiovascular response to thromboxane A_2. *Science* **296**:539-541.

Cocks TM, King SJ, Angus JA (1990). Glibenclamide is a competitive antagonist of the thromboxane A2 receptor in dog coronary artery in vitro. *Br J Pharmacol* **100**:375-378.

Collins R, Baigent C, Sandercock P, Peto R (1994). Antiplatelet therapy for thromboprophylaxis: the need for careful consideration of the evidence from randomised trials. Antiplatelet Trialists' Collaboration. *BMJ* **309**:1215-1217.

de Leval X, Hanson J, David JL, Masereel, Pirotte B, Dogné JM. New developments on thromboxane and prostacyclin modulators. Part II: Prostacyclin modulators. *Curr Med Chem*, in press.

Dogné JM, de Leval X, Hanson J, Frederich M, Lambermont B, Ghuysen A, Casini A, Masereel B, Ruan KH, Pirotte B and Kolh P. New developments on thromboxane and prostacyclin modulators. Part I: Thromboxane modulators. *Curr Med Chem*, in press.

Dogné JM, de Leval X, Kolh P, Sanna V, Rolin S, Michaux C, Mauer M, David JL, Masereel B, Pirotte B (2003). Pharmacological evaluation of the novel thromboxane modulator BM-567 (I/II). Effects of BM-567 on platelet function. *Prostaglandins Leukot Essent Fatty Acids* **68**:49-54.

Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S (2002). Aspirin-resistant

thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation* **105**:1650-1655.

Fiddler GI, Lumley P (1990). Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers. A review. *Circulation* **81**:I69-78.

Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ Jr, Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA, Chintala MS (2001). Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* **107**:1591-1598.

Gorelick PB, Richardson D, Kelly M, Ruland S, Hung E, Harris Y, Kittner S, Leurgans S (2003). African American Antiplatelet Stroke Prevention Study Investigators. Aspirin and ticlopidine for prevention of recurrent stroke in black patients: a randomized trial. *JAMA* **289**:2947-2957.

Hamberg M, Svensson J, Samuelsson B (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A* **72**:2994-2998.

Hamm CW, Lorenz RL, Bleifeld W, Kupper W, Wober W, Weber PC (1987). Biochemical evidence of platelet activation in patients with persistent unstable angina. *J Am Coll Cardiol* **10**:998-1006.

Kim YS, Pyo MK, Park KM, Park PH, Hahn BS, Wu SJ, Yun-Choi HS (1998). Antiplatelet and antithrombotic effects of a combination of ticlopidine and ginkgo biloba ext (EGb 761). *Thromb Res* **91**:33-38.

Koba S, Pakala R, Watanabe T, Katagiri T, Benedict CR (2000). Synergistic interaction between thromboxane A_2 and mildly oxidized low density lipoproteins on vascular smooth muscle cell proliferation. *Prostaglandins Leukot Essent Fatty Acids* **63**:329-335.

Kurz KD, Main BW, Sandusky GE (1990). Rat model of arterial thrombosis induced by ferric

chloride. *Thromb Res* **60**:269-80.

Lebrun P, Arkhammar P, Antoine MH, Nguyen QA, Hansen JB, Pirotte B (2000). A potent diazoxide analogue activating ATP-sensitive K+ channels and inhibiting insulin release. *Diabetologia* **43**:723-732.

Li P, Ferrario CM, Brosnihan KB (1998). Losartan inhibits thromboxane A_2-induced platelet aggregation and vascular constriction in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **32**:198-205.

Loï C, Chardigny JM, Berdeaux O, Vatele JM, Poullain D, Noel JP, Sebedio JL (1998). Effects of three trans isomers of eicosapentaenoic acid on rat platelet aggregation and arachidonic acid metabolism. *Thromb Haemost* **80**:656-661.

Ma H, Hara A, Xiao CY, Okada Y, Takahata O, Nakaya K, Sugimoto Y, Ichikawa A, Narumiya S, Ushikubi F (2001). Increased bleeding tendency and decreased susceptibility to thromboembolism in mice lacking the prostaglandin E receptor subtype EP(3). *Circulation* **104**:1176-1180.

Mangano DT; Multicenter Study of Perioperative Ischemia Research Group (2002). Aspirin and mortality from coronary bypass surgery. *N Engl J Med* **347**:1309-1317.

Masereel B, Schynts M, Krzesinski JM, Pirotte B, Rorive G, Delarge J (1993). A sulphonylthiourea (BM 20) related to torasemide: a new loop diuretic with relative potassium-sparing properties. *J Pharm Pharmacol* **45**:720-724.

Pakala R, Benedict CR (1998). Effect of serotonin and thromboxane A_2 on endothelial cell proliferation: effect of specific receptor antagonists. *J Lab Clin Med* **131**:527-37.

Pinsky PF, Hurwitz ES, Schonberger LB, Gunn WJ (1988). Reye's syndrome and aspirin. Evidence for a dose-response effect. *JAMA.* **260**:657-661.

Rolin S, Dogne JM, Michaux C, Delarge J, Masereel B (2001). Activity of a novel dual thromboxane A(2)receptor antagonist and thromboxane synthase inhibitor (BM-573) on

platelet function and isolated smooth muscles. *Prostaglandins Leukot Essent Fatty Acids* **65**:67-72.

Saldeen TG, Saldeen P, Nichols WW, Lawson DL, Nicolini FA, Mehta JL (1993). Increased production of thromboxane A_2 by coronary arteries after thrombolysis. *Am Heart J* **125**:277-284.

Stanke F, Cracowski JL, Chavanon O, Magne JL, Blin D, Bessard G, Devillier P (1998). Glibenclamide inhibits thromboxane A_2-induced contraction in human internal mammary artery and saphenous vein. *Eur J Pharmacol* **341**:65-71.

Svensson J, Hamberg M, Samuelsson B (1976). On the formation and effects of thromboxane A2 in human platelets. *Acta Physiol Scand* **98**:285-294.

Tanaka T, Ito S, Higashino R, Fukuta Y, Fukuda Y, Takei M, Kurimoto T, Tamaki H (1998). A new thromboxane receptor antagonist, Z-335, ameliorates experimental thrombosis without prolonging the rat tail bleeding time. *Thromb Res* **91**:229-35.

Tanaka T, Sato R, Kurimoto T (2000). Z-335, a new thromboxane A(2) receptor antagonist, prevents arterial thrombosis induced by ferric chloride in rats. *Eur J Pharmacol* **401**:413-418.

Uchida T, Kido H, Yamanaga K, Okita M, Watanabe M (1992). A novel loop diuretic, torasemide, inhibits thromboxane A_2-induced contraction in the isolated canine coronary artery. *Prostaglandins Leukot Essent Fatty Acids* **45**:121-124.

Wittner M, Di Stefano A, Schlatter E, Delarge J, Greger R (1986). Torasemide inhibits NaCl reabsorption in the thick ascending limb of the loop of Henle. *Pflugers Arch* **407**:611-614.

Wittner M, Di Stefano A, Wangemann P, Delarge J, Liegeois JF, Greger R (1987). Analogues of torasemide--structure function relationships--experiments in the thick ascending limb of the loop of Henle of rabbit nephron. *Pflugers Arch* **408**:54-62.

Yokoyama K, Kudo I, Nakamura H, Inoue K (1994). A possible role for extracellular bicarbonate in U-46619-induced rat platelet aggregation. *Thromb Res* 74:369-376.

Footnotes

This work is supported by FRSM (Fonds de la Recherche Scientifique Médicale) Grant no. 3.4.505.01.F. Vincent Tchana-Sato is Research Fellow of the FNRS (Fonds National de la Recherche Scientifique) and Laurence de Leval is a Research Associate of the FNRS.

Legends

Figure 1.

Arachidonic acid cascad, cyclooxygenase pathway. TXA_2 is formed by the action of thromboxane synthase on the prostaglandin endoperoxide H_2 (PGH_2) mainly in activated platelets where this enzyme is highly expressed. In platelets, PGH_2 is the result of the enzymatic action of the constitutive form of the cyclooxygenase (COX-1) on AA released from the cell membrane phospholipids by phospholipase A_2. In endothelial cells, prostacyclin synthase can convert PGH_2 into prostacyclin or prostaglandin I_2 (PGI_2).

Figure 2.

Chemical structures of the pyridinic sulfonylurea torasemide and the nitrobenzenic sulfonylurea BM-573.

Figure 3.

Time-dependent effect of BM-573 on platelet aggregation induced in rat washed platelets by U-46619. Each column represents the mean+/-SEM of 6-7 rats. * $P < 0.05$, compared to the vehicle-treated group.

Figure 4.

Blood levels of BM-573 (µg/ml) determined by HPLC. Mean+/-SEM, n=6, * $P < 0.05$, compared to the vehicle-treated group.

Figure 5.

A. Longitudinal section of an aorta showing a subocclusive thrombus developed induced by $FeCl_3$ (brown linear deposit, arrow)(hematoxylin and eosin, original magnification x50).

B. High-power view of the thrombus showing a meshwork of fibrin (arrows) associated with large aggregates of platelets (P) and red blood cells (RBC) (hematoxylin and eosin, original magnification x200).

Figure 6.

A. Dose-dependent effects of BM-573 on thrombus weight (mg/kg) 1 hour (white columns) and 2 hours (grey columns) before ferric chloride application on rat abdominal aorta.

B. Effects of seratrodast, torasemide and furegrelate (5 mg/kg, IP) on thrombus weight (mg/kg) 1 hour (white columns) and 2 hours (grey columns) before ferric chloride application on rat abdominal aorta. Each column represents the mean+/-SEM of 6-7 rats. * $P < 0.05$, compared to the vehicle-treated group.

Figure 7.

Effect of BM-573, furegrelate, seratrodast, acetylsalicylic acid and ticlopidine (5 mg/kg, IP, 1h pre-treatment) on tail bleeding time in mice. Mean+/-SEM, n=6, * $P < 0.05$, compared to the vehicle-treated group.

Figure 8.

Diuresis (ml/kg) measured in a time –dependent study in rats pretreated by a single dose (5 mg/kg) of BM-573 or torasemide. Columns represent the mean+/-SEM of the cumulated urine volumes, n=9, *$P < 0.05$, compared to the vehicle-treated group.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.063610
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 9.

Time courses of the changes in blood glucose level in fed rats injected intraperitoneally at time zero with BM-573 (20 mg/kg), glibenclamide (20 mg/kg) or the vehicle. Mean+/-SEM, n=6, * $P < 0.05$, compared to the vehicle-treated group.

Figure 1.

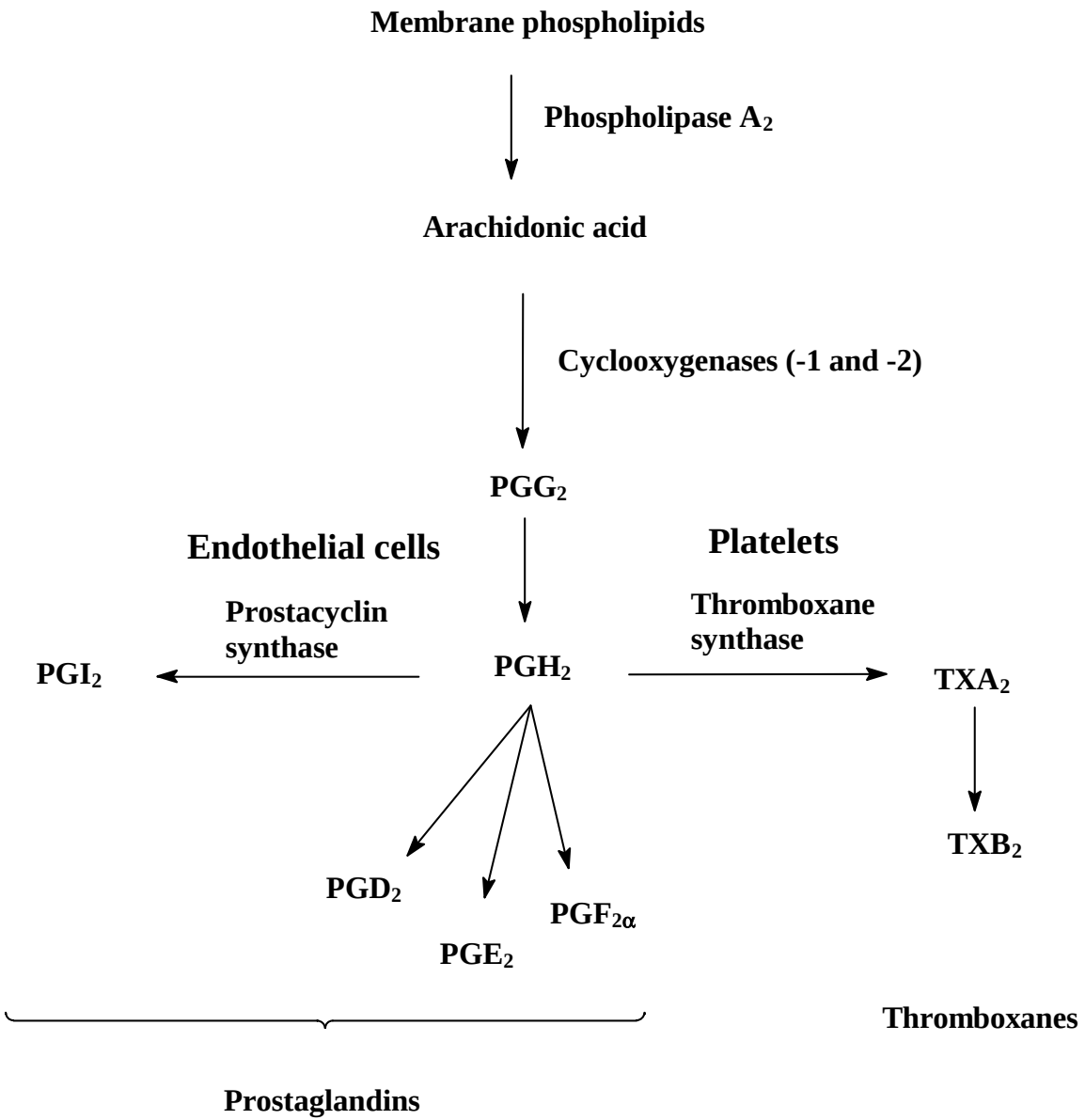

Figure 2

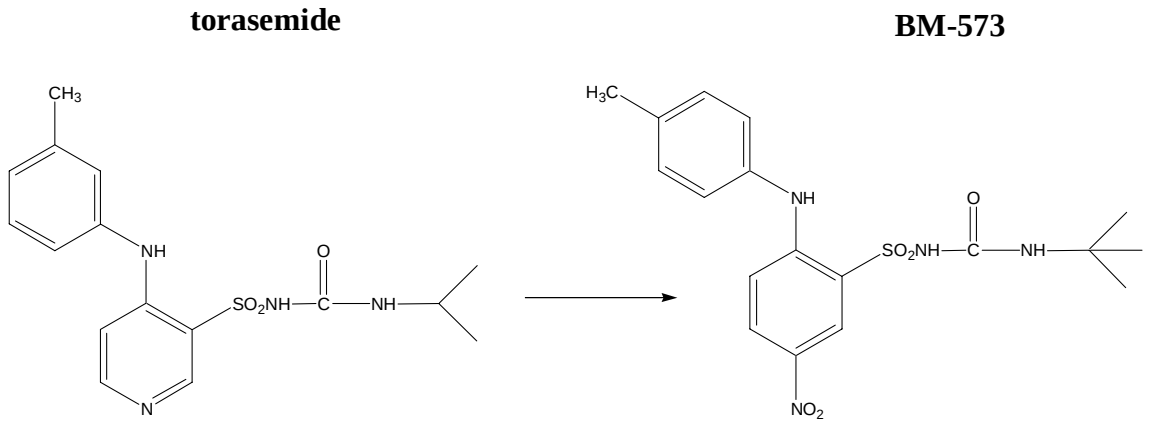

Figure 3

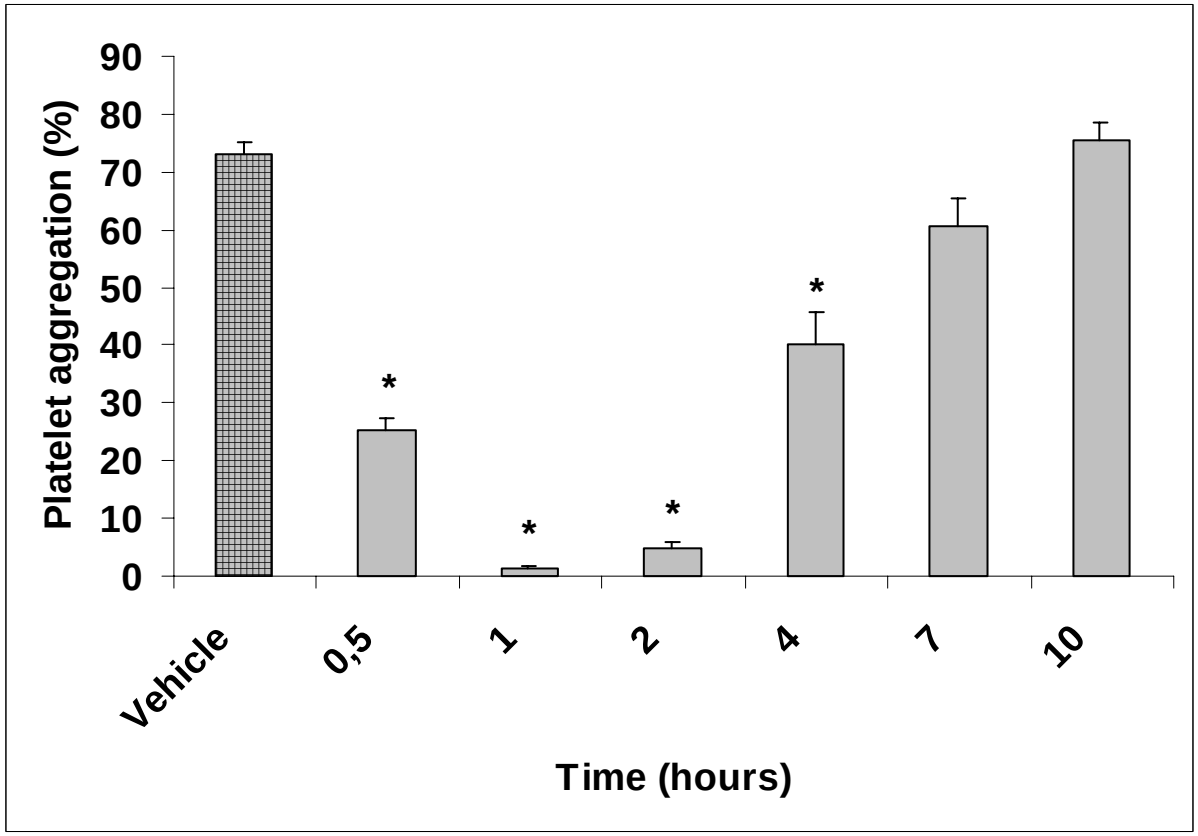

Figure 4

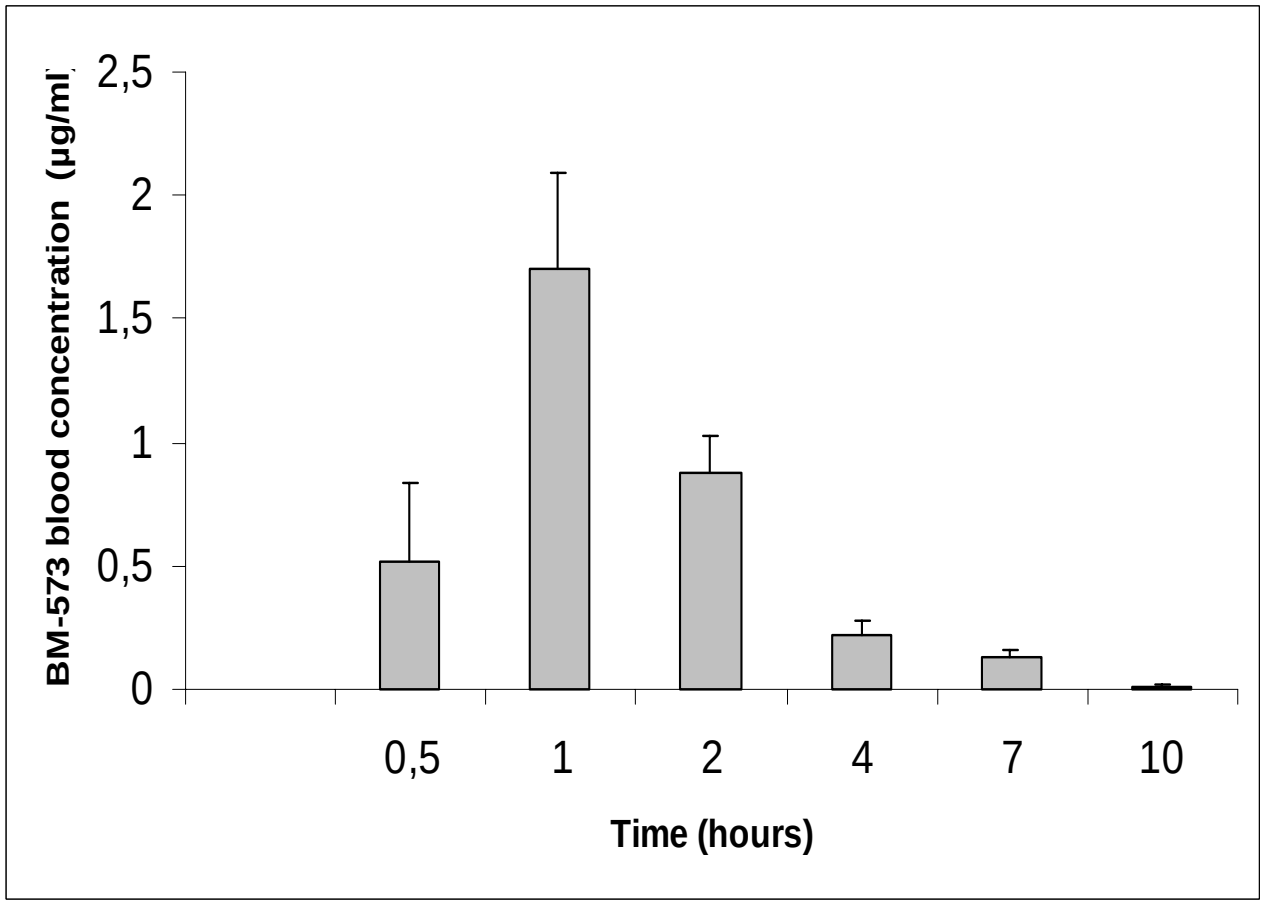

Figure 5.

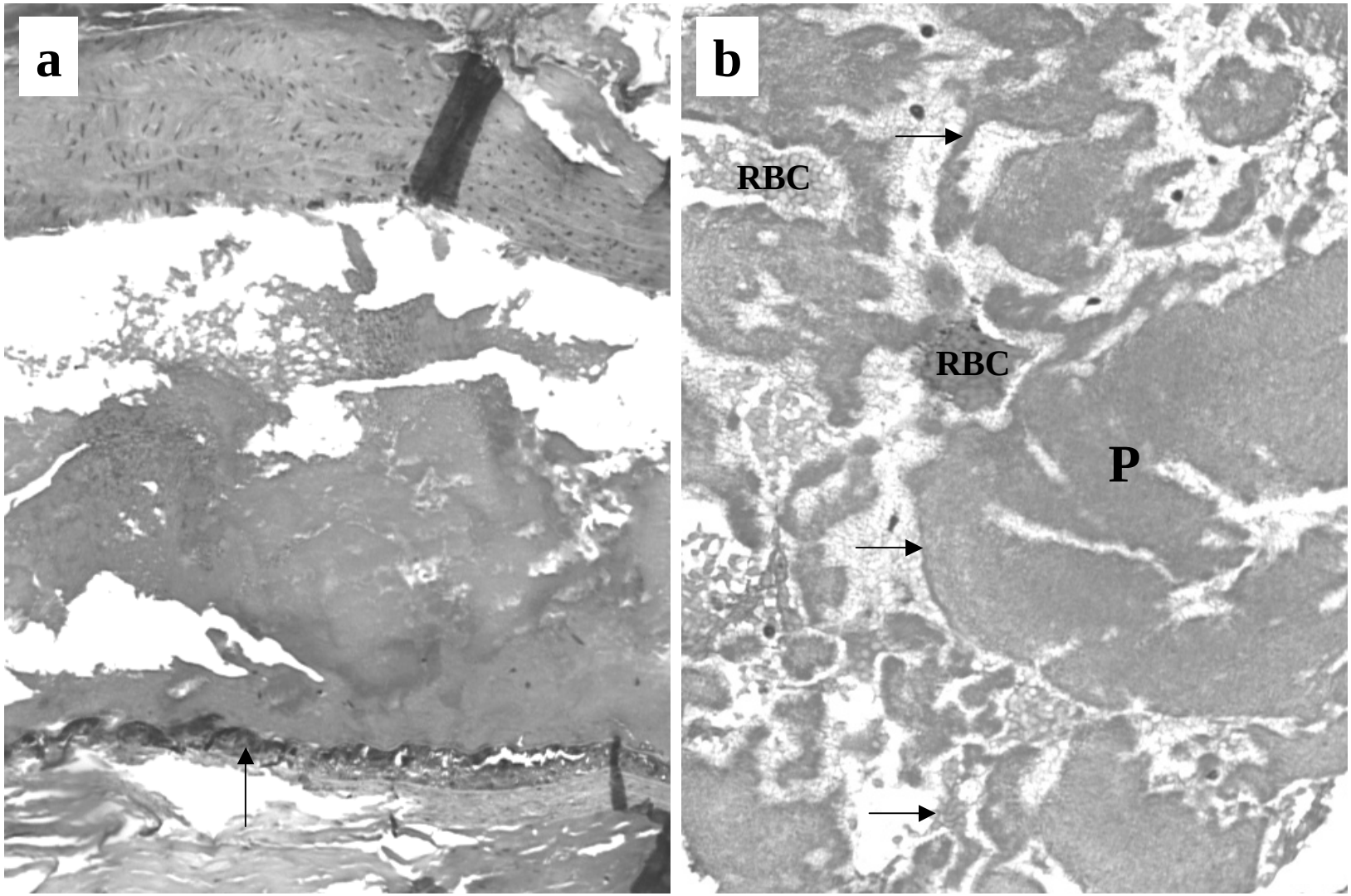

Figure 6

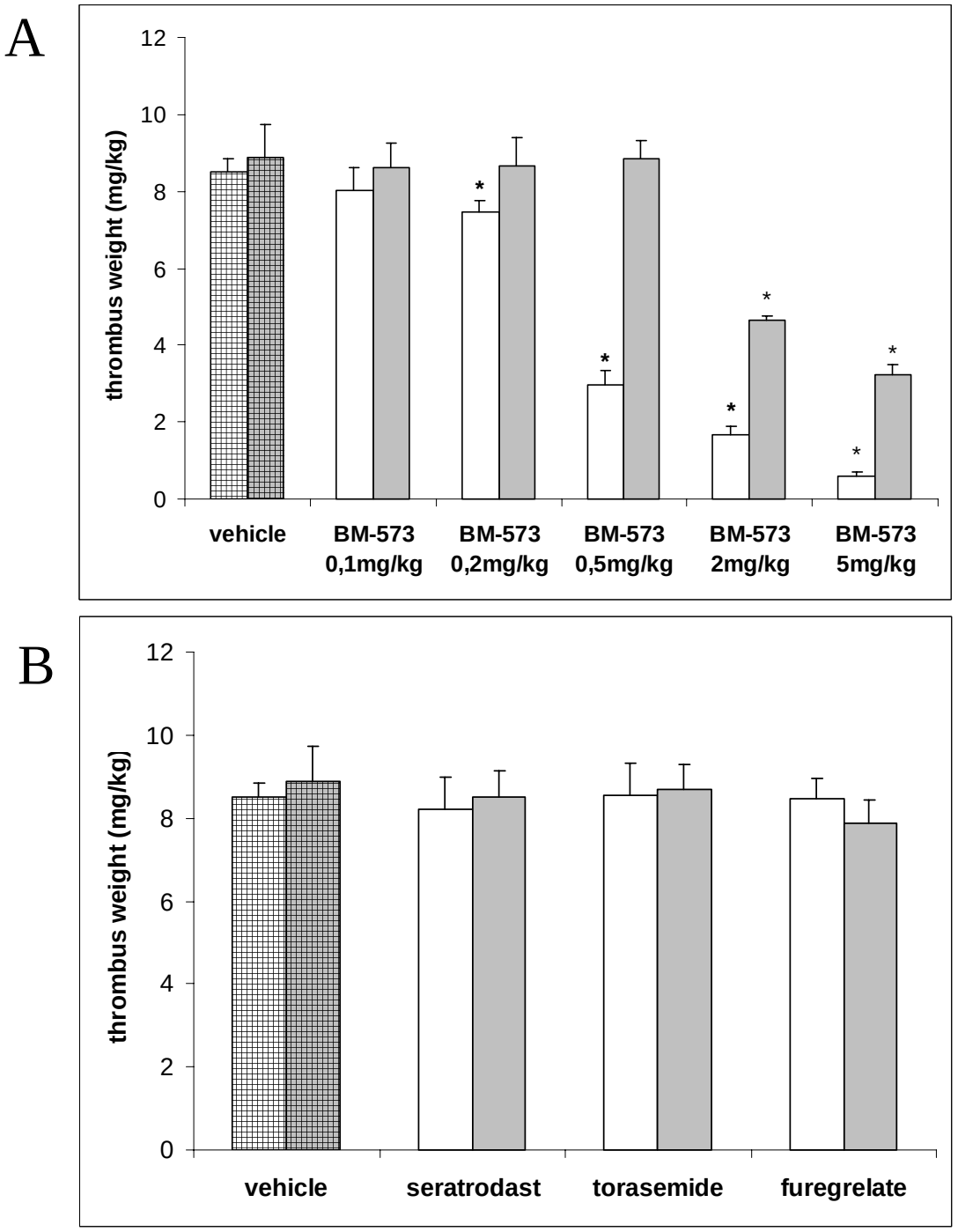

Figure 7

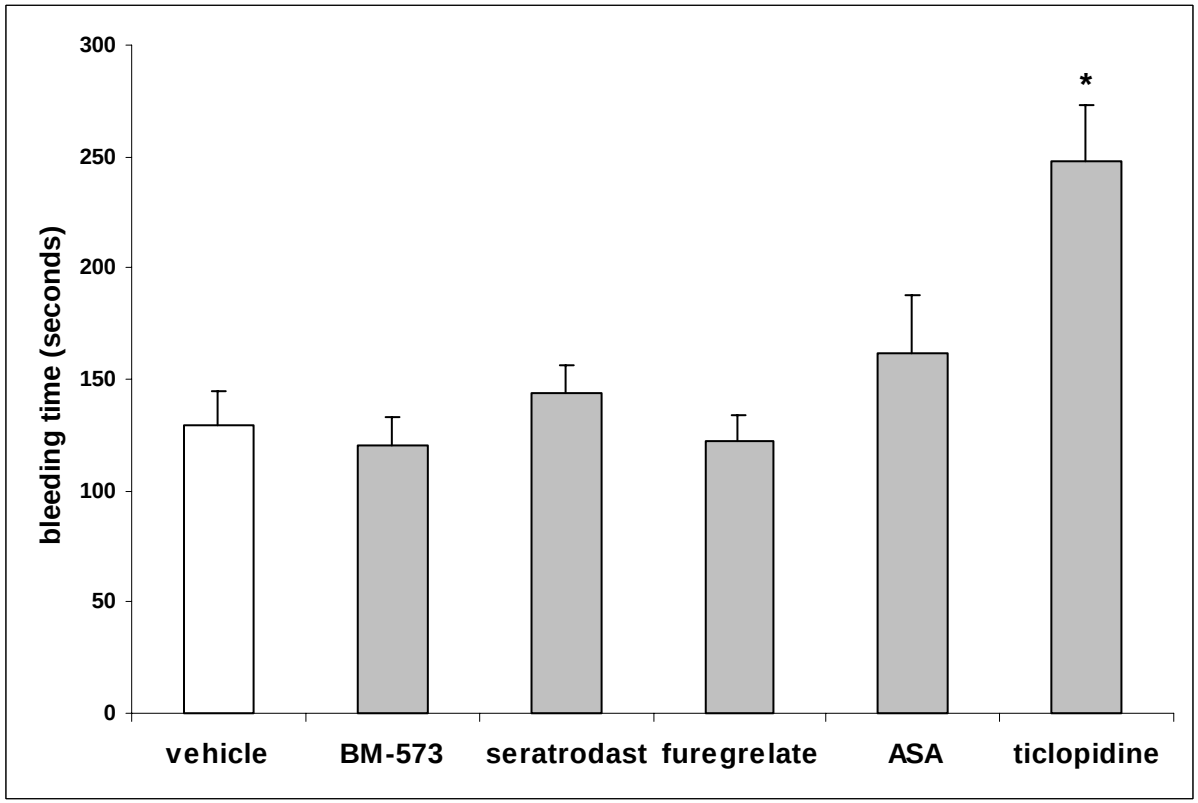

Figure 8

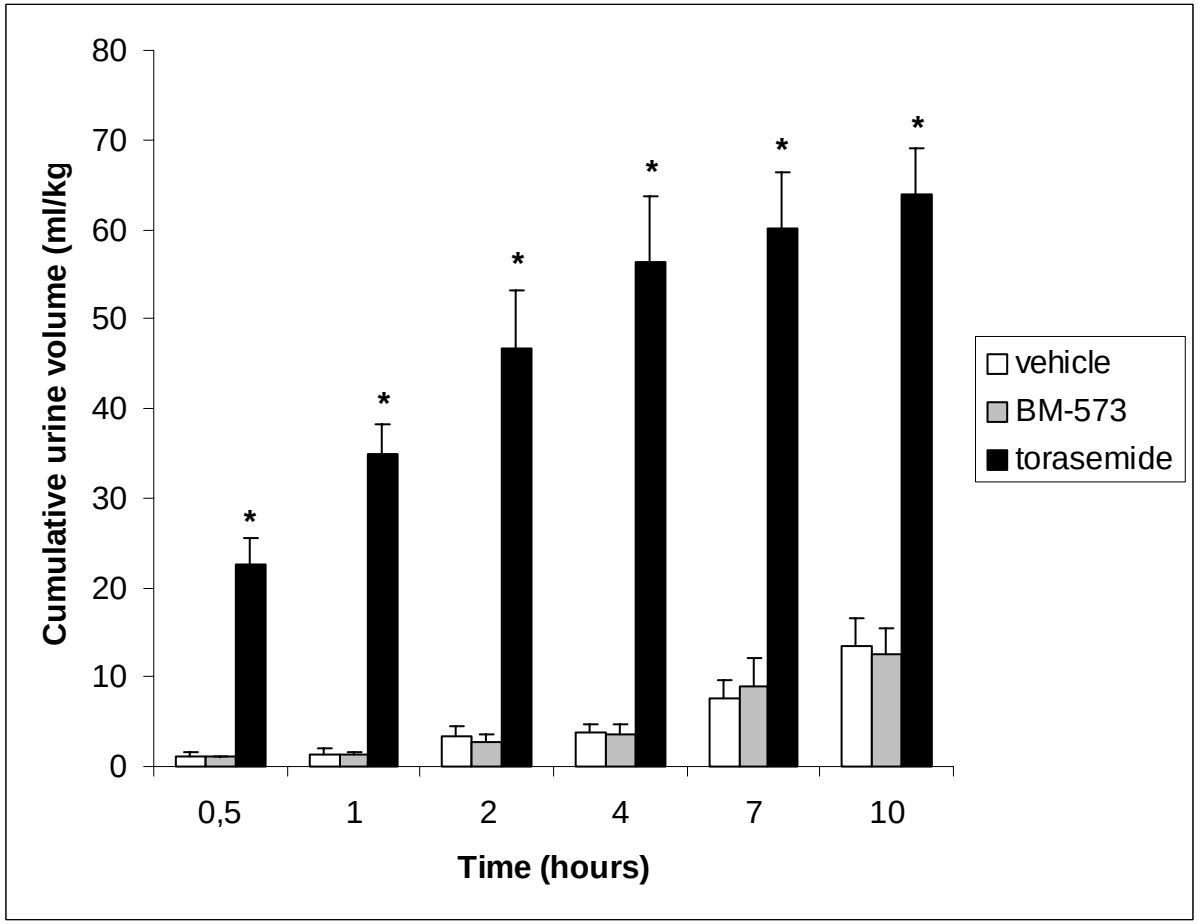

Figure 9