BM-573, a Dual Thromboxane Synthase Inhibitor and Thromboxane Receptor Antagonist, Prevents Pig Myocardial Infarction Induced by Coronary Thrombosis

S. ROLIN, M. PETEIN, V. TCHANA-SATO, J. M. DOGNE, P. BENOIT, B. LAMBERMONT, A. GHUYSSEN, P. KOLH, and B. MASEREEL

Department of Pharmacy, University of Namur, Namur, Belgium (S.R., B.M.); Institute of Pathology and Genetics, Loveland, Belgium (M.P.); Experimental Hemodynamics Laboratory (HemoLiège), University Hospital of Liège, Liège, Belgium (V.T.-S., B.L., A.G., P.K.); and Natural and Synthetic Drug Research Center, University of Liège, Liège, Belgium (J.M.D., P.B.).

Received November 6, 2002; accepted March 27, 2003

ABSTRACT

The aim of this study was to characterize the effects of BM-573 [N-terbutyl-N’-[2-(4’-methylphenylamino)-5-nitro-benzenesulfonyl] urea], a novel dual thromboxane A2 receptor antagonist and thromboxane synthase inhibitor, on myocardial infarction induced by topical ferric chloride (FeCl3) application to the left anterior descending (LAD) coronary artery in anesthetized pigs. All control animals (n = 6) developed an occlusive thrombus in the LAD coronary artery. The mean infarct size, revealed by triphenyl tetrazolium chloride; TnT, troponine T; LV, left ventricular; AUC, area under the curve.

Cardiovascular pathologies that remain the leading cause of mortality and morbidity in Western society include several diseases, such as ischemic cardiopathy of which myocardial infarction represents the most important form. Ischemic cardiopathy is characterized by an inadequacy between supply and demand in oxygenated blood correlated with a diminution of coronary blood flow due to coronary artery stenosis or occlusion. This artery occlusion is often caused by atherosclerotic lesions, acute thrombosis, edema, ballooning of atheromatous plaque, or bleeding (Pearson et al., 1977; Horie et al., 1978; Köenig, 2001). The consequence of this mismatch is necrosis characterized by the loss of cardiac myocytes (Hillis and Braunwald, 1977). After the onset of myocardial ischemia, it takes 6 h before myocardial necrosis can be identified by standard microscopic postmortem examination (Bouchardy and Majno, 1974; Van Reempts et al., 1976; Kloner et al., 1979).

In myocardial ischemia due to plaque rupture or thrombosis, it has been shown that thromboxane A2 (TXA2) and other platelet vasoactive mediators are released to promote platelet aggregation and vasoconstriction (Hirsh et al., 1981, 1983; Fitzgerald et al., 1986). TXA2, a short-live lipidic mediator generated by the cyclooxygenase pathway, is mainly produced by platelets, macrophages, and lung parenchyma (Hamberg et al., 1975; Moncada and Vane, 1979). TXA2 is a potent platelet activator and constrictor of vascular and bronchial smooth muscles. It has been demonstrated that drugs able to antagonize TXA2 receptors or to inhibit thromboxane synthase (TS) reduce the severity of myocardial ischemia (Schror et al., 1980; Burke et al., 1983; Hock et al., 1986;
Brezinsky et al., 1987). Thus, molecules combining a dual activity, such as TS inhibition and TXA₂ receptor antagonism, have a greater therapeutic interest since they reduce TXA₂ production and prevent the action of TXA₂ and PGH₂ (De Clerck et al., 1989a,b). As torasemide, a loop diuretic (Friedel and Buckley, 1991), showed a weak TXA₂ antagonist activity on dog coronary artery (UCHida et al., 1992), several chemically related molecules were designed and studied for their TXA₂ antagonism (Masereel et al., 1999; Dogné et al., 2000; Dogné et al., 2001). Recently, BM-573 [N-terbutyl-N'-(2-[4'-methylphenylamino]-5-nitro-benzenesulfonyl) urea], a torasemide-related molecule (Fig. 1), which lost the diuretic properties in rat, showed a high affinity for the human platelet TXA₂ receptor (IC₅₀: 1.3 nM). BM-573 relaxed the rat aorta contracted by U-46619, a TXA₂ agonist (ED₅₀: 28.4 nM). Moreover, BM-573 prevented human platelet aggregation induced by arachidonic acid (ED₁₀₀: 0.13 μM) and completely inhibited TS at 1 μM (Rolin et al., 2001).

In this work, we evaluated the activity of this novel TXA₂ antagonist in a model of myocardial ischemia evoked by thrombosis of the left anterior descending (LAD) coronary artery induced by ferric chloride in pig. To diagnose myocardial infarction, we applied histochemistry of mitochondrial dehydrogenases (Anderson et al., 1979; Fishbein et al., 1981). This method using 2,3,5-triphenyltetrazolium chloride (TTC) allows to reveal and quantify the area of necrosis. TTC stains undamaged myocardium, which appears brick red due to the presence of intact dehydrogenase system that reduces TTC in undamaged myocardium, which appears brick red due to the presence of intact dehydrogenase system that reduces TTC in insoluble-colored pigments called formazans (Nachlas and Shnittka, 1963). In necrotic tissue, the dehydrogenase system collapsed, and the area of necrosis is unstained. Samples from infarcted and normal regions were then studied by conventional histological analysis (hematoxylin and eosin, trichrome staining, and luxol fast blue) and immunohistochemical identification of desmin, an intermediate filament protein of myocardial muscle cells (Vargas et al., 1999). ATP dosage in normal and infarct regions were also realized for BM-573-treated pigs and compared with the control animals. Troponin T (TnT), a constitutive protein of myofibrils, is a regulator of muscular contraction and an efficient biological marker in myocardial infarction. TnT levels in plasma have been measured to investigate the effect of BM-573 on the increase of TnT due to myocardial infarction.

Materials and Methods

Surgical Procedure. All experimental procedures and protocols used in this investigation were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Liege. They were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Council of the American Physiological Society.

Experiments were performed on 18 healthy pure Pietran pigs of either sex weighing from 20 to 30 kg. The animals were premedicated with intramuscular administration of ketamine (20 mg · kg⁻¹) and diazepam (1 mg · kg⁻¹). Anesthesia was then induced and maintained by a continuous infusion of sufentanil (0.5 μg · kg⁻¹ · h⁻¹) and pentobarbital (5 mg · kg⁻¹ · h⁻¹). Spontaneous movements were prevented by pancuronium bromide (0.2 mg · kg⁻¹). After endotracheal intubation through a cervical tracheostomy, the pigs were connected to a volume cycled ventilator (Evita2; Dräger, Lübeck, Germany) set to deliver a tidal volume of 15 ml · kg⁻¹ with a FiO₂ of 0.4 and at a respiratory rate of 20 breaths · min⁻¹. End-tidal CO₂ (ET CO₂) measurements (Capnomac, Datex, Helsinki, Finland) were used to monitor the adequacy of ventilation. Respiratory settings were adjusted to maintain ET CO₂ between 30 and 35 mm Hg. Blood pressure and heart rate were measured via a catheter inserted into the ascending aorta. A catheter inserted into the right femoral artery was used for blood sampling. Jugular vein was cannulated for drug administration. Body temperature was monitored and kept at 37°C throughout the experiment using a heating blanket.

The heart was exposed through a median sternotomy. A segment of the LAD coronary artery was isolated distal to the first diagonal branch for thrombus formation. An electromagnetic flow probe (Transonic, Ithaca, NY) was placed at the distal end of the isolated LAD artery to measure the coronary artery blood flow.

Thrombus Induction. After a 30-min stabilization period, a blood sample was drawn (time 0) and a tissue strip (3 mm width) saturated with ferric chloride solution (50% w/v) was rolled around the LAD coronary artery for 45 min. The animals were randomized in three groups: a sham-operated control group (n = 6) intravenously infused with BM-573 (10 mg · kg⁻¹ · h⁻¹), a control group (n = 6) intravenously infused with vehicle (propylene glycol-NaCl 0.9%, 30–70 μl · kg⁻¹ · h⁻¹) and a BM-573-treated group (n = 6) infused with BM-573 (10 mg · kg⁻¹ · h⁻¹). The infusion started 30 min before ferric chloride application and was continuously infused till 6 h after strip application. When it occurred, the complete occlusion of LAD coronary artery was confirmed by the drop of coronary artery blood flow till 0, which appeared within 15 to 25 min.

Quantification of Area at Risk. The risk area and the infarct area were delineated by a dual staining technique (Warltier et al., 1981; Toki et al., 1988; Higo et al., 1992). Just before the end of the experiment, 20 ml of Evans blue dye solution (0.1 g · ml⁻¹ in 50 mM phosphate-buffered saline, pH 7.4) was injected into the jugular vein to stain the non ischemic area blue. The pig was then sacrificed with an injection of potassium chloride. LAD coronary artery was isolated and fixed with 10% formalin in phosphate buffer for 1 week. The heart was then rapidly removed and sectioned in five transverse slices (0.6 cm thick) from apex to base (S1 to S5). The risk area of left ventricle, due to its anatomical dependence on the LAD coronary artery for blood flow, was identified by lack of the Evans blue in this region. Gross slices have been photographed with a digital camera (Fujiﬁlm FinePix 2400 Zoom; Elmsford, NY).

Quantification of Myocardial Infarction. Morphological changes in size, shape, and transmural distribution of myocardial infarction were measured using TTC staining (Sigma-Aldrich, St. Louis, MO). Tissue slices were rinsed with a cold isotonic saline solution and then incubated at 38°C for 15 to 20 min in a phosphate-
buffered solution of TTC (1% in 0.1 M, pH 7.4) (Lie et al., 1975; Fishbein et al., 1981). This produced a brick red coloration in the presence of dehydrogenase enzymes in intact myocardium, whereas infarcted regions remained unstained due to the collapse of enzyme activity. After photography with a digital camera (Fujifilm FinePix 2400 Zoom), slices of myocardium were placed in 10% neutral-buffered formaldehyde to enhance the contrast between stained and unstained regions. After 3 days of fixation in formalin and just before paraffin proceeding, myocardial sections were still photographed with a video camera (3CCD color video, DXC-390P ExwaveHAD; Sony, New York, NY). Infarct size was measured from the tracings of these photographed myocardial slices on to a clear acetate sheet, and the area of each zone was then weighted. The ratio between the two zones (ischemic area and nonischemic area) was determined for each slice. Infarct size measured from the tracings of myocardial slices was calculated by planimetry as a percentage of LV mass.

**Microscopic analysis.** After 3 days, three or four tissue blocks were taken from each slice at standardized locations and routinely processed for paraffin histology. The tissue blocks from myocardium were cut at 0.6 μm, and each section was stained with hematoxylin and eosin, Masson's trichrome, and luxol fast blue. Sections obtained from the artery were stained with hematoxylin and eosin. The stained sections were examined at magnifications of 25×, 100×, 200×, and 400× to study the distribution of infarction. Photomicrographs were taken using a Zeiss photomicroscope (Axioscope 2 plus; Sony 3CCD camera, 1024–768 pixels definition; Thornwood, NY). In addition, all tissue-blocks of slice S3 were processed for immunohistochemical staining of desmin to investigate cardiac muscle's lesions. Monoclonal antibodies to desmin (clone D33; Biomeda, Foster City, CA) were diluted 1/20.

**ATP dosage in myocardial tissue.** The ATP content was measured by an adaptation of the ATP bioluminescent assay kit (Sigma-Aldrich diagnostic kit). There is a linear relationship between the relative light intensity generated by luciferin-luciferase reaction and the ATP concentration. Samples of cardiac tissue taken from anteroseptal and posterior regions of slice S2 were sequentially frozen in liquid nitrogen and then conserved at −80°C till ATP dosage. Sample was mechanically disrupted, reduced in powder, and then suspended in 700 μl of somatic cell ATP releasing reagent. The suspension was then homogenized with a Dounce (10 times), diluted with ultra pure water, and 10 μl added to the 100-μl ATP assay mix (luciferase, luciferin, MgSO₄, EDTA, dithiothreitol, and bovine serum albumin in a Tricine buffer). For each sample, luminescence measured in relative light units was determined with a luminometer (LUMAC Biocounter M2010). Bradford's protein dosage was performed, and results expressed in nanomoles of ATP per grams of protein.

**Troponine T Dosage in Plasma.** Arterial blood samples were taken at 0, 3, and 6 h after inducing LAD occlusion with ferric chloride. Collected samples were centrifuged at 2205g for 10 min at 15°C. The supernatant was removed, and the plasma concentration of TnT was measured (Lauer et al., 1997). The area under the curve (AUC) is expressed in micrograms per milliliter per 6 h.

**Drugs.** BM-573, synthesized in our laboratory, was dissolved in propylene glycol and administered at a concentration of 20 mg/ml.

**Statistics.** All data are expressed as the mean ± S.E.M. For analysis of the infarct size, tissue ATP activity and plasma troponin T levels, a Student's t test was used. P values <0.01 were considered as statistically significant.

**Fig. 2.** Mean aortic blood pressure (A), heart rate (B), and their product (C) of control (O), BM-573-treated animals (■), and sham-operated control animals (▲). Mean ± S.E.M., n = 6 for each group.
Results

Heart Rate and Mean Aortic Blood Pressure. Before FeCl₃ application, mean aortic blood pressure (Fig. 2A), heart rate (Fig. 2B), and their product (Fig. 2C) were not affected by infusion of BM-573. While heart rate increased from 110 to 150 beats min⁻¹ in control and BM-573-treated group, there was no significant difference in heart rate, mean aortic blood pressure, or in their product between both groups throughout the experiment. In the BM-573-treated group, two pigs presented a necrotic region due to the LAD artery occlusion (Fig. 3). For each pig that developed myocardial infarction, the infarct size, expressed as the percentage of LV mass, was calculated by planimetry from heart slices (S1 to S5). For each slice (S1 to S5), the mean infarct size was similar in control group and in both treated pigs that developed myocardial infarction (Table 1). The mean infarct size reported to the LV mass was similar for controls and both BM-573-treated pigs (35.3 ± 2.1 and 33.8%, respectively). The risk area has been revealed by Evans blue and then calculated for both groups. The mean risk areas calculated for the animals in control group and for the BM-573-treated pigs that developed an infarction were, respectively, 36.9 ± 2.1 and 34.8%. For each animal with occluded LAD artery, this risk area was superimposed to the necrotic zone revealed by TTC staining (Table 1).

Histopathological Examination of Infarcted Myocardium. Heart from six control animals was examined by light microscopy. The main ischemic changes were observed in the anterior left ventricle, corresponding to the area supplied by the LAD artery. Pyknosis of nuclei from muscle fibers, edema, and beginning leucodiapedesis from the capillaries suggested that these cells reached the stage of necrosis. If histological diagnosis could be established with hematoxylin and eosin staining, delineation between ischemic and non-ischemic region was unclear. For this reason, desmin immunohistochemical staining was used to detect microscopically the necrotic zone. Loss of staining with antibodies to desmin was detected in necrotic myocardium and closely related to the affected area revealed by TTC staining. In healthy regions from control and BM-573-treated pigs, positive immunohistological staining of desmin revealed the Z bands of cardiac muscle cells giving a typical striated appearance (Fig. 4A). The pattern and distribution of infarction identified histologically was related to the results obtained by TTC staining (Fig. 4B). In the sham-operated group, no histopathological changes were observed.

Histopathological Examination of Ferric Chloride-Injured Coronary Artery. Topical application of ferric chloride on the LAD artery induced an occlusive thrombus adherent to the vessel wall from the site of ferric chloride contact (Fig. 5A). Treatment with BM-573 partially prevented thrombus but did not protect against vascular injury due to ferric chloride. The three layers that constitute healthy arterial wall (intima, media, and adventice) were damaged at artery application site of ferric chloride (Fig. 5B).

ATP Dosage in Myocardium. ATP content was measured in myocardium from control and BM-573-treated animals. Two samples were judiciously taken from slice 2 of each heart, one in the anteroseptal region and one in the posterior region stained by TTC (Fig. 6).
In all control animals with an occlusive thrombus, a significant \((P < 0.01)\) myocardial ATP decrease was observed in the necrotic region (11630 ± 3410 versus 76.6 ± 43.6 nmol · g\(^{-1}\) of protein). Compared with the healthy region, a drop of ATP content was also measured in the necrotic area of both BM-573-treated animals, which presented LAD coronary artery occlusion (28,460 ± 10,140; 94.1 ± 33.4 nmol · g\(^{-1}\) of protein, respectively, \(P < 0.01\)). When BM-573 prevented LAD artery occlusion \((n = 4)\), ATP contents were similar in healthy and LAD artery supplied zones (9,484 ± 2,880; 16,760 ± 4,310 nmol · g\(^{-1}\) of protein, respectively), as observed in the sham-operated control group (27,590 ± 4,781; 27,140 ± 4,721 nmol · g\(^{-1}\) of protein, respectively).

**Troponine T Levels in Plasma.** Plasma levels of TnT were measured from control and BM-573-treated animals. A marked elevation of TnT concentration was observed in control group during 6 h of ischemia, whereas a significantly smaller rise of plasmatic TnT was seen when BM-573 prevented the LAD artery occlusion \((P = 0.0017)\). AUC were 2924 ± 278 and 656 ± 244 μg · ml\(^{-1}\) · 6h\(^{-1}\), respectively.

**Discussion**

In previous in vitro and ex vivo studies, it has been demonstrated that BM-573 was a potent dual compound able to reduce TXA\(_2\) production by TS inhibition and to prevent the action of TXA\(_2\) (or PGH\(_2\)) by blocking the TXA\(_2\) receptors.
TXA2 and PGH2 binding, they do not increase prostacyclin (Watanabe et al., 1989; Ruggeri, 1994). This could explain why the LAD artery occluded in two treated pigs.

To evaluate the myocardial protective effects of BM-573, the TTC staining method was used. This technique has been validated and represents a methodology for the early differentiation of infarcted and healthy myocardium. TTC staining can be used 2 h following coronary occlusion and after reperfusion (Fishbein et al., 1981). This technique relies on reduction of the colorless TTC salt by dehydrogenase enzymes to brightly colored red compounds in healthy tissue during a rather short incubation. In this study, the risk area was determined by intravenous injection of Evans blue 6 h after thrombus induction.

Even if similar anatomical sites were chosen to place ferric chloride, the risk area and the infarct size depend on heart size, on coronary anatomy, and on development of the coronary collateral circulation. In each pig with an occlusive thrombus, the size of the risk area determined by Evans’ blue staining was similar and superimposed to the infarct zone revealed by TTC, which could be attributed to the pig coronary collateral system being less developed than that of humans, and that is the reason why necrosis progression only occurred after 6 to 12 h in humans. Since risk area and infarct region are similar, the infarct size was expressed as a percentage of left ventricle. For the six control animals, occlusion of the LAD provoked by ferric chloride at the same anatomical site produced an infarct of 35.3 ± 2.1% of LV mass. Despite BM-573 infusion, two animals developed a LAD artery occlusion, and their infarct size was of 33.8% of LV mass.

Histopathological examination revealed myocardial necrosis largely pronounced in vehicle-treated group. This group, suffering 6 h of ischemia, showed evidence of the effect of anoxia with several alterations consisting of interstitial edema, granularity, small leukocytes infiltrates, anisokaryosis, and caryolysis. The infarct was clearly delineated by the lack of TTC staining, and there was a close correspondence between gross and histological measurements of necrosis area in each slice. Moreover, clear delineation between healthy and ischemic myocardium revealed on gross slices was clearly seen in desmin-stained sections and could be superimposed. Positive immunological staining of desmin revealed healthy myocardium with a high organization level in muscle cells taken from heart of BM-573-treated pigs that did not develop infarction. Each sample taken from the necrotic area showed a negative desmin staining and a loss of organization of myocardial tissue.

Following ischemia, the drop of ATP content is an indicator of tissue necrosis. A significant ATP depletion was measured for the control group, as for both treated pigs in which the LAD artery occluded. When BM-573 prevented LAD artery occlusion, no ATP change was observed, and the energetic intermediate level of myocardium was preserved. Plasma dosage of TnT, a sensitive and specific biological marker of necrosis, revealed a decrease of 77% of the AUC when BM-573 prevented ischemia compared with the control group.

In conclusion, we previously showed that BM-573, a dual TXA2 receptor antagonist and TS inhibitor prevents the formation of an occlusive thrombus in the LAD coronary artery without affecting heart rate, mean aortic blood pressure, and their product. Indeed, intravenous infusion of BM-573 (10 mg·kg⁻¹·h⁻¹) 30 min before thrombus induction in the LAD coronary artery prevents any myocardial tissue damage in four of six treated pigs. As observed for TS inhibitors (Simpson et al., 1987), BM-573 probably prevented the development of myocardial infarction by shunting PGH2 production to the synthesis of cardioprotective prostacyclin. On the other hand, if competitive TXA2 receptor antagonists prevent both TXA2 and PGH2 binding, they do not increase prostacyclin formation (Gresele et al., 1984). Thus, the combination of these two properties in BM-573 could be considered more beneficial in myocardial ischemic injury than molecules bearing only one of these features, TS inhibitor or TXA2 receptor antagonist.

The antiaggregant properties of BM-573 (Rolin et al., 2001) prevent the formation of an occlusive thrombus. When platelet aggregation is initiated, this phenomenon is irreversible and leads to a complete aggregation (“all or none phenomenon” (Son et al., 1987)). BM-573 probably prevented the development of coronary collateral circulation. In each pig with an occlusive thrombus, the size of the risk area determined by Evans’ blue staining was similar and superimposed to the infarct zone revealed by TTC, which could be attributed to the pig coronary collateral system being less developed than that of humans, and that is the reason why necrosis progression only occurred after 6 to 12 h in humans. Since risk area and infarct region are similar, the infarct size was expressed as a percentage of left ventricle. For the six control animals, occlusion of the LAD provoked by ferric chloride at the same anatomical site produced an infarct of 35.3 ± 2.1% of LV mass. Despite BM-573 infusion, two animals developed a LAD artery occlusion, and their infarct size was of 33.8% of LV mass.

Histopathological examination revealed myocardial necrosis largely pronounced in vehicle-treated group. This group, suffering 6 h of ischemia, showed evidence of the effect of anoxia with several alterations consisting of interstitial edema, granularity, small leukocytes infiltrates, anisokaryosis, and caryolysis. The infarct was clearly delineated by the lack of TTC staining, and there was a close correspondence between gross and histological measurements of necrosis area in each slice. Moreover, clear delineation between healthy and ischemic myocardium revealed on gross slices was clearly seen in desmin-stained sections and could be superimposed. Positive immunological staining of desmin revealed healthy myocardium with a high organization level in muscle cells taken from heart of BM-573-treated pigs that did not develop infarction. Each sample taken from the necrotic area showed a negative desmin staining and a loss of organization of myocardial tissue.

Following ischemia, the drop of ATP content is an indicator of tissue necrosis. A significant ATP depletion was measured for the control group, as for both treated pigs in which the LAD artery occluded. When BM-573 prevented LAD artery occlusion, no ATP change was observed, and the energetic intermediate level of myocardium was preserved. Plasma dosage of TnT, a sensitive and specific biological marker of necrosis, revealed a decrease of 77% of the AUC when BM-573 prevented ischemia compared with the control group.

In conclusion, we previously showed that BM-573, a ligand of the human platelet TXA2 receptor (IC50 1.3 nM), was able to relax a rat aorta precontracted with U-46619 (ED50 28.4 nM), to prevent human platelet aggregation induced by arachidonic acid (ED100 0.13 μM), and to completely inhibit TS at 1 μM. In this study, we demonstrated that BM-573, a dual
TS inhibitor and TXA2 receptor antagonist, was able to prevent the formation of an occlusive thrombus in the LAD artery of BM-573-treated pigs. They did not develop infarction, and their myocardium was totally healthy with a protected structure despite attempt to form thrombus in the coronary artery. The coronary thrombus of each vehicle-treated pig was adherent and occlusive. As expected, BM-573 did not protect against vascular injury due to ferric chloride. These data suggest that BM-573 could be useful in acute coronary syndrome and during coronary angioplasty. Further experiments using an ischemia-reperfusion model will contribute to establish the clinical interest of this dual TS inhibitor and TXA2 antagonist.

Acknowledgments
We thank Dr. Michaela Oana for advice on this experiment, Carine Michiels for helping in ATP dosage, and Cécile Meraglia for excellent technical assistance.

References

Address correspondence to: Prof. Bernard Masereel, Department of Pharmacy, University of Namur, 61, rue de Bruxelles, 5000 Namur, Belgium. E-mail: bernard.masereel@unamur.be