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Thromboxane A_2 Receptor Antagonism in Man and Rat by a Sulphonylcyanoguanidine (BM-144) and a Sulphonylurea (BM-500)

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Abstract

Torasemide, a loop diuretic, has been reported to relax dog coronary artery precontracted by thromboxane A_2 (TXA_2), an endogenous prostanoid involved in cardiovascular and pulmonary diseases. *N*-cyano-*N*′-{[4-(3′-methylphenylamino)pyrid-3-yl]sulphonyl}homopiperidinoamidine (BM-144) and *N*-isopropyl-*N*′-[5-nitro-2-(3′-methylphenylamino)-benzenesulphonyl]urea (BM-500), chemically related to torasemide, have been examined for their TXA_2 antagonism.

The affinity (IC50, the concentration resulting in 50% inhibition) of BM-144 and BM-500 for the TXA_2 receptor of washed platelets from man was 0·28 and 0·079 μM, respectively. This is better than for sulotroban (IC50 = 0·93 μM) but less than for SQ-29548 (IC50 = 0·021 μM), two TXA_2 antagonists used as reference. The aggregation of platelets from man induced by arachidonic acid was prevented by BM-144 (IC50 = 9·0 μM) and by BM-500 (IC50 = 14·2 μM). Similar results were obtained by use of U-46619, a TXA agonist, as aggregating agent (BM-144, IC50 = 12·9 μM and BM-500, IC50 = 9·9 μM). The contracting effect of U-46619 on rat stomach strip was abolished by BM-144 (IC50 = 1·01 μM) and BM-500 (IC50 = 2·54 μM). Both drugs (BM-144: IC50 = 0·12 μM and BM-500: IC50 = 0·19 μM) also relaxed rat aorta precontracted by U-46619; both were more potent than sulotroban (IC50 = 1·62 μM). The two torasemide derivatives (100 μM) did not significantly affect the myo-stimulating effect of some prostaglandins (PGE_2, PGI_2, $PGF_{2\alpha}$) or aorta contraction elicited by KCl (30 mM). They did not modify rat diuresis after administration of a 30-mg kg^{-1} dose.

In conclusion, BM-144 and BM-500 can be regarded as novel non-carboxylic TXA_2 receptor antagonists and offer a novel template for the design of more potent molecules.

Thromboxane A_2 (TXA_2) and its precursor, the endoperoxide prostaglandin H_2 (PGH_2), are potent inducers of vasoconstriction, bronchoconstriction and platelet-aggregation, acting at a common receptor site (Charo et al 1977; Coleman et al 1981; Bhagwat et al 1985). They are involved in the aetiology and pathology of cardiovascular (myocardial infarction), renal (hypertension) and pulmonary (asthma) diseases (Fitzgerald et al 1986; Oates et al 1988a, b ; Coleman & Sheldrich 1989). Therapeutically, inhibition of the effects of TXA_2 results from inhibition of TXA_2 synthase or from the blocking of TXA_2 receptors. Many TXA_2 synthase inhibitors have been developed, but clinical trials with these agents have been very disappointing, probably because they lead to accumulation of PGH_2 which has a similar biological profile to that of TXA_2. Many TXA_2 antagonists have been synthesized and can be chemically classified into three groups: analogues of TXA_2 or

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PGH_2, exemplified by SQ-29548 (Ogletree et al 1985), one of the most potent TXA_2 antagonists (Figure 1); the sulphon-amide derivatives, of which sulotroban (Gresele et al 1984) can be considered the prototype (Figure 1); and a variety of tricyclic derivatives (Collington & Finch 1990; Jakubowski et al 1992). In the same way as TXA_2, all these drugs are characterized by a carboxylic function which probably interacts with Arg-295 of the TXA_2 receptor (Yamamoto et al 1993). In 1992, torasemide (Figure 1) was reported to weakly inhibit the TXA_2-induced contraction of isolated canine coronary artery (Uchida et al 1992). This drug is a loop-diuretic with a sulphonylurea function which acts by inhibiting the $Na^+/2Cl^-/K^+$ cotransporter located on the luminal membrane of Henle's loop (Friedel & Buckley 1991). Several molecules structurally related to torasemide have been described for their diuretic (Masereel et al 1993, 1995), antihypertensive (Masereel et al 1992), neuroprotective (Masereel et al 1994) or anticonvulsive (Masereel et al 1997, 1998) properties. With the aim of discovering potent and selective TXA_2 antagonists we have investigated the TXA_2 antagonism of *N*-cyano- *N*′-{[4-(3′-methylphenylamino) pyrid - 3 - yl]sulphonyl}homopiperidinoamidine (BM-144) and *N*-isopropyl- *N*′-[5-nitro-2-(3′-methylphenylamino)benzenesulphonyl]urea (BM-500), structurally related to torasemide (Figure 1). The affinity of these two compounds for the TXA_2 receptor of washed platelets from man was studied. Their activity was evaluated on platelet aggregation induced by arachidonic acid, the natural precursor of TXA_2, by U-46619, a TXA_2 agonist, by ADP or by collagen. The antagonism of both compounds against the myo-stimulating effect of U-46619 on rat stomach strip and rat aortic ring was also examined. Finally, we investigated the specificity of the inhibitory activity of these novel TXA_2 antagonists.

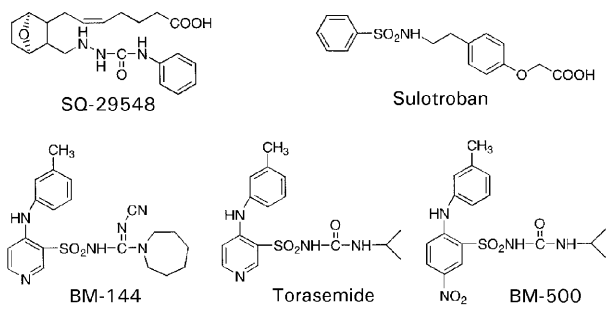

Figure 1. The chemical structure of SQ-29548 and sulotroban, and of torasemide and its two derivatives (BM-144 and BM-500).

Materials and Methods

Drugs and chemicals

Torasemide and its derivatives were synthesized according to general synthetic pathways previously reported (Masereel et al 1994, 1995). Their elemental analyses for C, H, N and S were performed with a Carlo-Erba (Milano, Italy) analyser and were within 0·4% of the theoretical values. Nuclear magnetic resonance (^{1}H NMR) and infrared (IR) spectra were recorded on a Jeol GX 400 MHz spectrometer (Bruker, Karlsruhe, Germany) and a Perkin-Elmer (Düsseldorf, Germany) 1750 spectrometer. They are in accordance with the proposed chemical structures. Concentrated drug solutions were prepared in dimethylsulphoxide (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 (5 mM; Sigma, Belgium) and adenosine diphosphate (100 μM, Stago, France) 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 as described elsewhere (Nuhrich et al 1996). The solution of collagen (25 μg mL^{-1}) was provided by Horm (France) and [^{3}H]SQ-29548 (46 Ci mmol^{-1}) by NEN Products (Brussels, Belgium).

Platelet binding

Platelet-rich plasma from man 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* and 4°C. The supernatant was removed and the pellet was resuspended in calcium- and magnesium-free Tyrode-Hepes buffer (mM: NaCl 137, KCl 2·7, NaH_2PO_4 0·4, $NaHCO_3$ 12, D-glucose 5, HEPES; pH 7·4) to a concentration of 2×10^8 cells mL^{-1}. Freshly prepared samples of this suspension (500 μL) were incubated with [^{3}H]SQ-29548 (5 nM final concn, 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-fibre 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 (10 mL) and the radioactivity was counted. The amount of [^{3}H]SQ-29548 specifically bound to the TXA receptor of platelets from man (B_s, %) was calculated from equation 1:

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

where B_t (total binding) and NSB (non-specific binding) are the radioactivity of [^{3}H]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 [^{3}H]SQ-29548 (5 nM) and the studied compound at a fixed concentration ranging from 10^{-5} to 10^{-10} M. In each experiment NSB varied between 5 and 7% of B_t. For each drug, three concentration–response curves were measured in triplicate (Figure 2). The concentration (IC50) which reduced the amount of specifically bound [^{3}H]SQ-29548 (B_s) by 50%, and its 95% confidence interval, were determined for each drug (Table 1) by non-linear regression analysis (GraphPad Prism software).

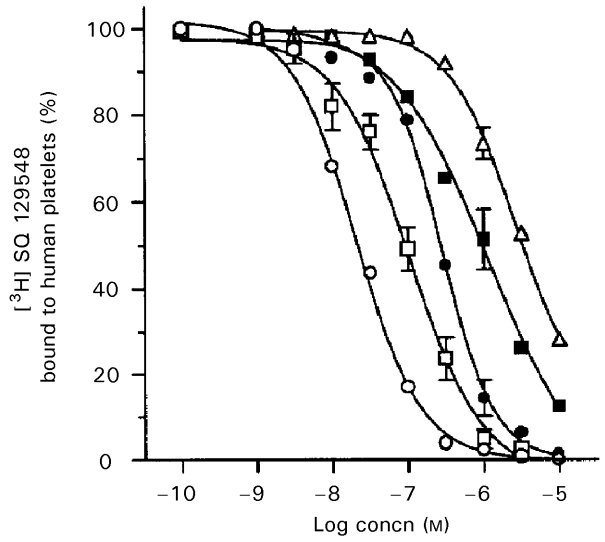

Figure 2. Displacement of [^{3}H]SQ-29548 from the TXA_2 receptor of washed platelets from man by SQ-29548 (○), sulotroban (■), torasemide (△) BM-500 (□), and BM-144 (●). Results are means ± s.e.m., n = 3.

Table 1. Drug concentration (IC50, μM) displacing 50% of [^{3}H]SQ-29548 specifically bound to the thromboxane A_2 receptor of washed platelets from man.

Drug	IC50*
SQ-29548	0·021 (0·020–0·022)
Sulotroban	0·93 (0·85–1·02)
Torasemide	2·69 (2·45–2·86)
BM-144	0·28 (0·22–0·34)
BM-500	0·079 (0·069–0·086)

*Calculated from at least three triplicate concentration–response curves by non-linear regression. Values in parentheses are 95% confidence intervals.

Platelet aggregation

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 platelet-rich plasma (PRP) was obtained from the supernatant fraction after centrifugation (90 *g*, 20 min) at 25°C. The remaining blood was centrifuged at 2000 *g* for 5 min and the supernatant gave the platelet-poor plasma (PPP). The platelet concentration of PRP was adjusted to 3–4 × 10^8 cells mL^{-1} by dilution with PPP. Aggregation tests were performed according to Born's turbidimetric method (Born & Cross 1963) by means of a four-channel aggregometer (BioData Corporation, PAP_4). 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 3 min. Platelet aggregation was initiated by addition of a convenient agent (5 μL)—arachidonic acid (60 μM final), U-46619 (1 μM final), ADP (1·6 μM) or collagen (1 μg mL^{-1}). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 min after addition of the inducer. The drug concentration (IC50) reducing platelet aggregation by 50%, and its 95% confidence interval, were calculated by non-linear regression analysis (GraphPad Prism software) from at least nine dose–response curves (Table 2).

Rat fundus contraction

Rat stomach was removed from fed male Wistar rats, 250–300 g, and fundus strips were prepared according to Vane (1957). The strips were set up in a 50-mL isolated organ bath at 37°C. The bathing fluid (mM: NaCl 137, KCl 2·7, NaH_2PO_4 0·4, $MgCl_2$ 0·5, $CaCl_2$ 1·8, $NaHCO_3$ 12, D-glucose 5) was continuously oxygenated with 95 : 5 O_2–CO_2. The strips were washed every 15 min and left to equilibrate in the bath for 45 min at a resting tension of 1 g recorded with an isotonic transducer coupled to a Harvard Universal oscillograph. A control contraction was induced by adding U-46619 (50 μL, 30 nM final). When the tension had stabilized, the preparation was rinsed twice. After recording three similar control responses and washing out, the TXA_2 potential antagonist (100 μL) was added to the bath 5 min before

Table 2. Drug concentration (IC50, μM) preventing 50% arachidonic acid-, U-46619-, ADP-, or collagen-induced aggregation of platelets from man.

Drug	IC50* (μM)			
	Arachidonic acid (60 μM)	U-46619 (1 μM)	ADP (1·6 μM)	Collagen (1 μg mL^{-1})
SQ-29548	0·034 (0·028–0·039)	0·035 (0·030–0·040)	0·010 (0·008–0·011)	0·046 (0·042–0·046)
Sulotroban	12·3 (9·6–15·4)	10·1 (8·3–11·9)	20·1 (16·2–24·6)	15·7 (11·2–21·4)
Torasemide	> 100	> 100	> 100	> 100
BM-144	9·0 (7·8–10·3)	12·9 (10·2–15·8)	30·4 (24·0–37·6)	122 (108–137)
BM-500	14·2 (13·5–14·9)	9·9 (8·3–11·8)	46·5 (39·3–54·6)	52·2 (36·4–72·6)

*Calculated by non-linear regression from three concentration–response curves determined in triplicate. Values in parentheses are 95% confidence intervals.

addition of U-46619 (50 μL). The response was recorded, the maximum tension measured and the results expressed as the percentage reduction of the maximum tension of a control response. The preparation was then washed twice, its quality checked by means of a control contraction with U-46619, washed twice again, and was then ready for study of the effect of another drug concentration on the prevention of fundus contraction evoked by U-46619. The fundus response to a U-46619 contraction is stable for at least 4 h, and the preparation was not used for times longer than this. For each drug, 3–4 concentration–response curves were acquired from strips isolated from different animals. The drug concentration (IC50) reducing the contractile activity of U-46619 by 50%, and its confidence interval 95%, were calculated by non-linear regression analysis (GraphPad Prism software) (Table 3). The same procedure was used to evaluate the efficacy of the novel drugs (100 μM) on the myo-stimulating activity induced by prostaglandins I_2 (PGI_2, 0·5 μM), E_2 (PGE_2, 0·05 μM) and $F_{2\alpha}$ ($PGF_{2\alpha}$, 0·1 μM).

Table 3. Drug concentration (IC50, μM) reducing by 50% the contractile activity of the rat fundus or aorta evoked by U-46619 (30 nM).

Drug	IC50*	
	Fundus	Aorta
SQ-29548	0·19 (0·14–0·25)	0·021 (0·0098–0·052)
Sulotroban	0·83 (0·81–1·79)	1·62 (1·59–1·64)
Torasemide	17·5%†	NA
BM-144	1·01 (0·88–1·15)	0·12 (0·07–0·17)
BM-500	2·54 (1·72–3·74)	0·19 (0·14–0·24)

*Calculated by non-linear regression from dose–response curves. Values in parentheses are 95% confidence intervals. †Relaxation (%) induced by 10 μM torasemide. NA = Not active at 10 μM.

Rat aorta contraction

Rat aortic rings were obtained from fed male Wistar rats, 250–300 g, and prepared as previously reported (De Tullio et al 1996). The endothelium was gently rubbed off and the segments were suspended under 1-g tension by means of steel hooks in an organ bath containing Krebs solution (20 mL; in mM: NaCl 118, KCl 4·7, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25, D-glucose 5) kept at 37°C and oxygenated continuously with 95 : 5 O_2–CO_2. The isometric contractions of the aortic rings were measured with a Grass force-displacement transducer. After 60 min equilibrium the rings were exposed to U-46619 (30 nM). When the tension was

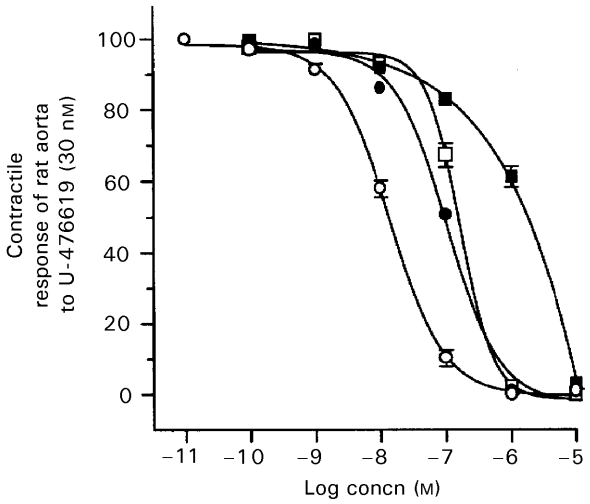

Figure 3. Concentration–relaxing activity of SQ-29548 (○), sulotroban (■), BM-500 (□) and BM-144 (●) on the rat aortic tonus evoked by U-46619 (30 nM). Means ± s.e.m. of 6–8 dose–response curves.

stable (10 min) the potential TXA_2 antagonist (50 μL) was added to the bath at cumulatively increasing concentrations until relaxation was maximal. The relaxation response was expressed as the percentage of the contractile response to U-46619. The IC50 value of each drug was assessed for 6–8 concentration–response curves obtained from separate preparations (Figure 3), and expressed as the concentration evoking 50% inhibition of the plateau induced by U-46619 (30 nM). The IC50 values, and their 95% confidence intervals, were calculated by non-linear regression analysis (GraphPad Prism software) (Table 3).

Diuresis

Groups of nine Wistar rats, 203–254 g, received a single oral or intraperitoneal dose of BM-144 or BM-500 (30 mg kg^{-1}) suspended in a volume of 40 ml kg^{-1}. NaCl (0·9%) was used as control. The rats were allowed free access to food and drink until the beginning of the experiment. The rats were housed in groups of three in metabolism cages. Urine was collected for 4 h after drug administration and diuresis (mL kg^{-1}) was expressed as mean ± s.d. of urinary volume collected from three cages.

Results and Discussion

In a first set of experiments, we determined the capacity of torasemide and its two derivatives to displace [^{3}H]SQ-29548, a potent competitive ligand of the TXA_2 receptor, from its binding site on platelets from man. SQ-29548 (Ogletree et al 1985) and sulotroban (Gresele et al 1984), two TXA_2 antagonists, were chosen as reference drugs. Their concentration–response curves (Figure 2) enabled calculation of their IC50 values (Table 1). The affinity of BM-500 was only a quarter that of SQ-29548. The cyanoguanidine BM-144 had an IC50 one third that of sulotroban (Table 1). Torasemide was confirmed to be a poor ligand for the TXA_2 receptor. The steepness of the slope obtained with BM-144 ($-1{\cdot}30 \pm 0{\cdot}19$) was significantly different ($P < 0{\cdot}05$) from those calculated for SQ-29548 ($-1{\cdot}00 \pm 0{\cdot}03$), sulotroban ($-0{\cdot}93 \pm 0{\cdot}15$), torasemide ($-0{\cdot}99 \pm 0{\cdot}16$) and BM-500 ($-0{\cdot}91 \pm 0{\cdot}18$). The slopes of the binding curves for SQ-29548 and sulotroban were similar to those previously described (Hedberg et al 1988; Theis et al 1992). Chemically, torasemide is a drug described as a weak TXA_2 antagonist lacking a carboxylic function. The replacement of the pyridine of toras-emide with a nitrobenzene ring (BM-500) strongly increased the affinity for the TXA_2 receptor of washed platelets from man (Figure 2 and Table 1). This affinity could be related to the nitro group which mimics the carboxylic function of TXA_2. In BM-144, the substitution of the urea group by a cyanoguanidine function and replacement of the isopropylamino with perhydroazepinyl, an unusual group, strengthened the affinity for the TXA_2 receptor.

Table 2 shows the efficacy of the drugs at inhibiting platelet aggregation induced by arachidonic acid (60 μM), U-46619 (1 μM), ADP (1·6 μM) or collagen (1 μg mL^{-1}) in platelet-rich plasma from man. As observed for the TXA_2 antagonists, the aggregation evoked by arachidonic acid was prevented dose-dependently by BM-144 and BM-500. Their IC50 values were similar to that of sulotroban. Torasemide was inactive and SQ-29548 was the most potent compound studied. Similar results were obtained by using the TXA_2 agonist U-46619 as aggregating agent. When ADP was used as inducer, none of the tested drugs prevented the initial phase of aggregation, known to be unaffected by TXA_2 synthase inhibitors or by TXA_2 receptor antagonists (data not shown). On the other hand, the prostaglandins derived from arachidonic acid are implicated in the secondary phase of ADP aggregation and in the collagen-induced response of platelets from man. On this second aggregation wave, sulotroban, BM-144 and BM-500 were much less active than SQ-29548. Unlike SQ-29548, sulotroban, BM-144 and BM-500 weakly prevented the platelet aggregation evoked by collagen. Torasemide (100 μM) was inactive, irrespective of the inducer used. Ignoring the magnitude of their activity, BM-144 and BM-500 had anti-aggregant profiles similar to those of the TXA_2 antagonists used as reference drugs. Surprisingly, the potency of BM-144 and BM-500 decreased when ADP or collagen were used as inducers. The reason for these discrepancies are unknown. The discrepancy between the IC50 values calculated from the binding test to the TXA_2 receptor of washed platelets from man and calculated from the aggregation experiment could be attributed to a strong affinity of the BM-derivatives for plasma proteins present in the aggregating test (Cozzi et al 1994; Soyka et al 1994). This phenomenon has also been reported for other sulphonylureas (Balant 1981).

In addition to their anti-aggregating activity, each drug prevented the contraction of rat fundus strips. This inhibition was concentration-dependent. The IC50 of BM-144 on the U-46619-induced contraction was similar to that of sulotroban but five times that of SQ-29548 (Table 3). BM-500 was one third as active as sulotroban ($P < 0{\cdot}01$) and

torasemide (10 μM) reduced the contraction evoked by U-46619 by only 17·5%. When added alone none of the five drugs had any myo-stimulating effect on rat stomach strips at the concentrations used (10^{-4} to 10^{-10} M). The specificity of both torasemide derivatives was examined by contracting the fundus strip with other prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGI_2, data not shown). At a high concentration (100 μM), BM-144 and BM-500 reduced PGI_2- (0·5 μM) induced contraction by $12·3 \pm 1·4\%$ and $9·3 \pm 1·7\%$ respectively. Only BM-144 (100 μM) reduced ($16·8 \pm 2·6\%$) the contraction evoked by PGE_2 (0·05 μM). BM-500, SQ-29548 and sulotroban had no effect. Used alone, none of these compounds reduced or amplified the intensity of the fundus contraction induced by $PGF_{2\alpha}$ (0·1 μM). Thus, BM-144 and BM-500 can be regarded as selective TXA_2 antagonists.

The TXA_2-antagonism of the two torasemide derivatives was also confirmed by their ability to relax rat aortic rings contracted by U-46619 (30 nM). The relaxing activity of SQ-29548, sulotroban, BM-144 and BM-500 is concentration-dependent (Figure 3). The calculated IC50 values (Table 3) showed that BM-144 and BM-500 were, respectively, one sixth and one ninth as potent as SQ-29548 but more active than sulotroban. On rat aorta both compounds were more efficient than on stomach strip. This could be explained by the heterogeneity of the TXA_2 receptors whose distinct subtypes have been described (Furci et al 1991; Krauss et al 1996). At 10 μM, torasemide had no significant effect. Up to 100 μM, none of the TXA_2 antagonists studied modified the aortic tonus sustained by KCl (30 mM).

Despite possessing chemical structures very similar to those of diuretics such as torasemide, BM-20 (Masereel et al 1993) and BM-106 (Masereel et al 1995), BM-144 and BM-500 failed to increase the volume of urine excreted by the rat in the 4 h after oral or intraperitoneal administration of 30 mg kg^{-1} (Table 4). It is of structural interest to note that some hypoglycaemic sulphonylureas have been reported to block the aorta contractions induced by U-46619 (Delaey & Van de Voorde 1995). Their phenylsulphonylurea group is identical to that of BM-500.

Table 4. Rat diuresis in the 4 h after oral or intraperitoneal administration of vehicle, torasemide, BM-144 or BM-500 at a dose of 30 mg kg^{-1}.

Treatment	Diuresis (mL kg^{-1})	
	Oral	Intraperitoneal
Control	$21·1 \pm 1·7$	$13·8 \pm 3·2$
Torasemide	$88·3 \pm 3·5$*	$82·0 \pm 3·0$*
BM-144	$20·0 \pm 2·0$	$15·3 \pm 2·6$
BM-500	$17·7 \pm 3·1$	$15·6 \pm 1·3$

Results are means ± s.d., nine rats per group. *$P < 0·001$ compared with the corresponding control.

In conclusion, BM-144 and BM-500 can be regarded as novel non-carboxylic TXA_2 receptor antagonists with chemical structures much different from those of sulotroban and SQ-29548, widely used TXA_2-antagonists with prostanoid structures. The chemical structures of BM-144 and BM-500 are novel templates for development of potent and original TXA_2 antagonists useful in cardiovascular pathology.

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