Evaluation of Original Dual Thromboxane A₂ Modulators as Antiangiogenic Agents

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ABSTRACT

Angiogenesis is a promising target for the therapy of several diseases including cancer. This study was undertaken to characterize the antiangiogenic properties of a series of original dual thromboxane A₂ (TXA₂) inhibitors derived from torasemide, a marketed loop diuretic with TXA₂ antagonistic properties, by evaluating their effects on human endothelial cell migration, adhesion, and viability in vitro, as well as in the ex vivo rat aortic ring assay. All drugs tested exhibited a marked affinity toward human platelet TXA₂ receptor, significantly prevented platelet aggregation induced by U-46,619, a stable TXA₂ receptor agonist, and inhibited platelet TXA₂ synthase without affecting cyclooxygenase (COX)-1 or COX-2 enzymatic activities. These dual TXA₂ inhibitors dose dependently inhibited endothelial cell migration in chemotaxis assays using vascular endothelial growth factor (VEGF) as a chemoattractant but failed to affect cell adhesion and viability. The highest rates of cell migration inhibition were obtained with original compounds BM-567 and BM-573 (50.3 and 59.4% inhibition, respectively) when used at the final concentration of 10 μM. In addition, pretreatment of endothelial cells with these two drugs significantly prevented U-46,619-induced intracellular Ca²⁺ pool mobilization, thus suggesting a mechanistic link between inhibition of the TXA₂ pathway and reduced endothelial cell migration. Treatment of rat aortic explants with U-46,619 (9,11-dideoxy-9,11-methanoepoxy-prostaglandin F₂) significantly enhanced vessel sprouting whereas aortic rings treated with some of the compounds, including BM-567 (N-n-pentyl-N'-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea) and BM-573 (N-tert-butyln-N'-[5-nitro-2-p-tolylaminobenzenesulfonyl]urea), showed a significant decrease in vessel sprouting, which was not reversed by the addition of VEGF. These data suggest that our original dual TXA₂ inhibitors bear antiangiogenic properties, mainly by inhibiting endothelial cell migration.

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X.D.L. and T.D. contributed equally to this work. B.P. and V.C. codirected the study.

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ABBREVIATIONS: COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; TXA₂, thromboxane A₂; VEGF, vascular endothelial growth factor; U46,619, 9,11-dideoxy-9,11-methanoepoxy-prostaglandin F₂; HUVEC, human umbilical endothelial cell; SQ-29,548, 1S-[1α,2α(2),3α,4α]-7-[3-[[2-[[phenylamino]carbonyl][hydrazinomethyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TXA₂ receptor; TXS, thromboxane synthase; BM-500, N-isopropyl-N'-[5-nitro-2-m-tolylaminobenzenesulfonyl]urea; BM-520, N-tert-butyl-N'-[5-nitro-2-m-tolylaminobenzenesulfonyl]urea; BM-531, N-tert-butyl-N'-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea; BM-567, N-n-pentyl-N'-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea; BM-573, N-tert-butyl-N'-[5-nitro-2-p-tolylaminobenzenesulfonyl]urea; BM-600, N-n-butyl-N'-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea; BM-616, N-tert-butyl-N'-[2-(4-bromo-3-methylphenylamino)-5-nitrobenzenesulfonyl]urea; BM-519, N-[homopiperidinocyanomino]carboxyl-5-nitro-2-m-tolylaminobenzenesulfonamide; BM-615, N-n-pentyl-N'-[2-(cyclohexylamino)-5-benzonitrilsulfonyl]urea; DMEM, Dulbecco’s modified Eagle’s medium; PRF, platelet-rich plasma; PPP, platelet-poor plasma; AA, arachidonic acid; PG, prostaglandin; PGI₂, prostacyclin.
pressed by quiescent endothelial cells, has been shown to be up-regulated in proliferative endothelial cells (Belton et al., 2000; Gately, 2000). Therefore, COX-2 inhibition has been considered as an interesting approach for the management of tumor-associated angiogenesis (Gately and Li, 2004). Accordingly, results from preclinical investigations have indicated that both selective COX-2 inhibitors and nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) may effectively reduce or inhibit tumor angiogenesis (Gately and Li, 2004; Wei et al., 2004). Although the vast majority of studies have provided compelling and converging evidence that COX-2 inhibition may protect against tumor angiogenesis, the implementation of cancer prevention strategies, based on these findings, is hampered by the recognized toxicity of chronic use of NSAIDs (Fortun and Hawkey, 2005). Furthermore, the recent withdrawal of specific COX-2 inhibitor Vioxx due to cardiovascular toxicity (Bresalier et al., 2005) represents a new serious obstacle to the targeting of COX-2 for cancer prevention and/or treatment (Vanchieri, 2004). Therefore, the selective targeting of downstream COX-2 pathway metabolites or enzymes may appear to be a promising approach with the potential of avoiding the cardiovascular toxicity of COX-2 inhibitors while maintaining their antiangiogenic properties. One of these downstream metabolites, TXA2, a powerful aggregation mediator involved in thrombotic disorders, has been recently demonstrated to participate in cancer progression (Nie et al., 2004; Moussa et al., 2005) and cancer cell-induced platelet aggregation, a process that favors blood-borne metastasis (Jurazi et al., 2004). On the other hand, TXA2 also acts as a potent angiogenesis stimulator, both directly and by inducing platelet vascular endothelium growth factor (VEGF) and platelet-derived growth factor secretion after platelet aggregation (Arisato et al., 2003; Rhee et al., 2004). Furthermore, in endothelial cells, VEGF and basic fibroblast growth factor have been shown to stimulate the biosynthesis of TXA2, which is correlated with enhanced cell migration (Nie et al., 2000). Finally, treatment of endothelial cells with U-46,619 (9,11-dideoxy-9,11-methanoep-2-yl-5-heptenoic acid), a TXA2 receptor (TP receptor) antagonist, fur- eralate [5-(3-pyridinylmethyl)-2-benzofuranacarboxylic acid, sodium salt], a TXA2 synthase (TXS) inhibitor and U-46,619, a stable TP recep-
tor agonist, were purchased from Cayman Chemical (Ann Arbor, MI). These compounds were dissolved in ethanol (furegrelate and SQ-29,548) or methyl acetate (U-46,619) and diluted to the required final concentration with saline. Original compounds synthesized in the Labor-atory of Medicinal Chemistry, University of Liège, Belgium (Fig. 1), were as follows: BM-500 (N-isopropyl-N’-[5-nitro-2-m-toluyaminobenzenesulfonyl]urea); BM-520 (N-tolyt-butyl-N’-[5-nitro-2-m-tolylaminobenzenesulfonyl]urea); BM-531 (N-tolyt-butyl-N’-[2-(cyclohexalamino)-5-nitrobenzenesulfonyl]urea); BM-567 (N-n-pentyl-N’-[2-(cyc- holoxymino)-5-nitrobenzenesulfonyl]urea); BM-573 (N-tolyt-butyl-N’-[5-nitro-2-p-toluyaminobenzenesulfonyl]urea); BM-600 (N-n-butyl-N’-[2-cyclohexylaminio)-5-nitrobenzenesulfonyl]urea); BM-616 (N-tolyt-butyl-N’-[2-(4-bromo-3-methylphenylamino)-5-nitrobenzenesulfonyl]urea); BM-519 (N-homopiperidinoyaminocarboxyl-5-nitro-2-m-toluyaminobenzenesulfonamide); BM-615 (N-n-pentyl-N’-[2-(cyclohexyminio)-5-benzonitrilsulfonyl]urea); and torasemide (N-isopropyl-N’-[4-m-toluyaminio-3-pyridyl]sulfonyl]urea). They were dissolved in dimethylsulfoxide and phosphate-buffered saline (30:70 v/v, respectively) to obtain 1 mM stock solutions. Further dilutions were performed using phosphate-buffered saline alone.

**Materials and Methods**

**Drugs and Reagents.** SQ-29,548 (1S-[1α,2α(Z)],3α,4α]-7-[3-[2-(phenylamino)carbonyl]hydrazine]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid), a TXA2 receptor (TP receptor) antagonist, fur- eralate [5-(3-pyridinylmethyl)-2-benzofuranacarboxylic acid, sodium salt], a TXA2 synthase (TXS) inhibitor and U-46,619, a stable TP recep-
tor agonist, were purchased from Cayman Chemical (Ann Arbor, MI). These compounds were dissolved in ethanol (furegrelate and SQ-29,548) or methyl acetate (U-46,619) and diluted to the required final concentration with saline. Original compounds synthesized in the Labor-atory of Medicinal Chemistry, University of Liège, Belgium (Fig. 1), were as follows: BM-500 (N-isopropyl-N’-[5-nitro-2-m-toluyaminobenzenesulfonyl]urea); BM-520 (N-tolyt-butyl-N’-[5-nitro-2-m-tolylaminobenzenesulfonyl]urea); BM-531 (N-tolyt-butyl-N’-[2-(cyclohexalamino)-5-nitrobenzenesulfonyl]urea); BM-567 (N-n-pentyl-N’-[2-(cycloholoxymino)-5-nitrobenzenesulfonyl]urea); BM-573 (N-tolyt-butyl-N’-[5-nitro-2-p-toluyaminobenzenesulfonyl]urea); BM-600 (N-n-butyl-N’-[2-cyclohexylaminio)-5-nitrobenzenesulfonyl]urea); BM-616 (N-tolyt-butyl-N’-[2-(4-bromo-3-methylphenylamino)-5-nitrobenzenesulfonyl]urea); BM-519 (N-homopiperidinoyaminocarboxyl-5-nitro-2-m-toluyaminobenzenesulfonamide); BM-615 (N-n-pentyl-N’-[2-(cyclohexyminio)-5-benzonitrilsulfonyl]urea); and torasemide (N-isopropyl-N’-[4-m-toluyaminio-3-pyridyl]sulfonyl]urea). They were dissolved in dimethylsulfoxide and phosphate-buffered saline (30:70 v/v, respectively) to obtain 1 mM stock solutions. Further dilutions were performed using phosphate-buffered saline alone.

**Cell Culture.** HUVECs (five donors pooled batch) were purchased from Biowhittaker (Petit-Rechain, Belgium), grown in endothelial growth medium-2 (EGM-2; Biowhittaker) according to the manufac-
turer’s instructions and used for pharmacologic experimentation at passage 3 to 6. Viability was determined by counting the number of cells failing to take up trypan blue dye. There was ≥95% viability in each experiment performed.

**Animals.** Eight- to 12-week-old male Wistar rats weighing 250 to 300 g were sacrificed by decapitation after i.p. injection of chloral hydrate (400 mg/kg). The thoracic aorta was removed under a dis-
section microscope and immediately transferred into culture dishes containing cold serum-free DMEM (Invitrogen, Grand Island, NY). The periaortic fibroadipose tissue was removed with microdissection forceps and iridectomy scissors, paying attention not to damage the aortic wall. One-millimeter-long aortic rings (~22–25 per thoracic aorta portion) were sectioned with a surgical blade and further rinsed in five consecutive cold serum-free DMEM baths. These experimental procedures were carried out in accordance with the Declaration of Helsinki (Principles of Laboratory Animal Care, National Institutes of Health publication 85-23, revised 1985) and had been reviewed and approved by the Ethics Committee of the University of Liège (Liège, Belgium).

**Human Platelet TP Receptor Affinity.** The binding test was performed using washed human platelets according to a method described previously (Dogne et al., 2003). The concentration of drug that reduced the amount of specifically bound [5,6-3H]SQ-29,548 by 50% (IC50) was determined for each drug by nonlinear regression analysis (GraphPad Prism software). Results were expressed as means ± S.D.

**In Vitro Human Platelet Aggregation.** Blood was collected by venipuncture from healthy volunteers reported to be free from any medication for at least 10 days and diluted (9:1) with trisodium citrate (3.8% w/v) in a polypropylene tube. Platelet-rich plasma (PRP) was obtained from the supernatant fraction after centrifugation for 10 min at 90g (25°C). The remaining fraction was further centrifuged at 2000g for 10 min (25°C), and the supernatant, a platelet-poor plasma (PPP), was harvested. The platelet concentra-
tion of PRP was determined using a hemacytometer and adjusted to 3 × 10^5 cells/μl by mixing with buffered saline. An aggregation test was performed according to Born’s turbidimetric method (Born and Cross, 1963) using a two-channel aggregometer (Chrono-log). PPP
was used to adjust the photometric measurement to the minimal optical density. PRP (225 μl) was added in a silanized cuvette and stirred (1100 rpm). Drug solution (25 μl) was then added, and the mixture was incubated at 37°C for 3 min. Platelet aggregation was initiated by addition of 5 μl of arachidonic acid (AA) (600 μM final concentration) or 2 μl of U-46,619 (1 μM final concentration). To evaluate platelet aggregation, the maximal increase in light transmission was determined from the aggregation curves 7 min after addition of the inducer. The drug concentrations able to decrease U-46,619-induced platelet aggregation by 50% (IC50) were calculated by nonlinear regression analysis from at least three concentration-response curves. Results were expressed as means ± S.D. of at least three independent experiments.

**Arachidonic Acid-Induced Platelet TXA2 Generation.** After incubation of PRP with arachidonic acid in the presence or absence of the drugs being investigated, platelet TXA2 generation was stopped by addition of indomethacin (100 μM final concentration). The PRP was then centrifuged, and supernatant TXB2 levels were measured as the stable metabolite of TXA2 by a specific enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions. Results are expressed as the means ± S.D. of at least three independent experiments.

**Purified Ovine COX-1 and COX-2 Enzyme Inhibition Test.** Purified ovine COX-1 and COX-2 inhibition assays were performed as described previously (de Leval et al., 2001) and prostaglandin (PG) E2 production was quantified by a specific enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions.

**HUVEC Chemotaxis Assay.** HUVECs were harvested from 70 to 80% confluent colonies and resuspended in endothelial basal medium-2 (Biowhittaker) containing 2% fetal bovine serum (Biowhittaker, Petit-Rechain, Belgium) at a density of 5 × 10^6 cells/ml. HUVEC migration was assessed using modified Boyden chambers (BD Biosciences, San Jose, CA). Cells were plated in the upper chamber, and migration was initiated by placing 1 ml of endothelial basal medium-2 containing 10 ng/ml VEGF (Biowhittaker) in the bottom chamber. Drugs to be investigated were added to both the upper and bottom chambers to obtain a 10 μM or 1 μM final concentration. After 12 h of incubation at 37°C in a humidified incubator with a 5% CO2-air atmosphere, the migration was stopped by replacement of the medium by methanol followed by a 20-min incubation at −20°C. Cells were stained in a 6% Giemsa solution in water. Cells at the upper side of the membrane were removed using a cotton swab, and the membrane was cut out and fixed. Cells that had migrated through the membrane were counted. For each experimental condition, a minimum of three chambers were used.

**HUVEC Intracellular Calcium Pool Mobilization.** The protocol used to assess intracellular calcium pool mobilization was performed essentially as described by Kroll et al. (2005). In brief, HUVECs were harvested from 70 to 80% confluent populations, washed twice with Krebs-HEPES buffer, and incubated for 1 h at 37°C in 10 ml of Krebs-HEPES buffer supplemented with 50 μg of Fluo-4 (Invitrogen, Eugene, OR). After incubation, the cell suspension was centrifuged and resuspended in Krebs-HEPES at a density of 5 × 10^6 cells/ml; 150 μl of Fluo-4-treated HUVEC suspension were added to each well of a 96-well plate and further incubated in a Fluoroskan Ascent (Thermo Electron Corporation, Waltham, MA) for 15 min in the presence or absence of BM-567 or BM-573 (10 μM final concentration). HUVEC intracellular Ca2+ influxes were measured fluorimetrically after the addition of U-46,619 (1 μM final concen-
is the Fluo-4 

Then, 2 

dium. After 20 h of incubation, cells were harvested and counted.

titer plates precoated with 0.2% (v/v) gelatin (Sigma, Bornem, Bel-

gium). Twenty-four hours later, medium was removed and replaced

with 20% fetal bovine serum (proliferation or viability, respec-

Values represent means ± S.D. from at least three independent experiments.

Assessment of Endothelial Cell Adhesion. HUVEC adhesion

tests were performed as described previously (Bellahcene et al., 2000). In brief, microtiter 96-well plates (Greiner, Wemmel, Bel-

gium) were coated with 10 μl of vitronectin (1 μg/ml) or fibronectin (60 μg/ml). HUVECs were incubated with various concentrations of test compound or with vehicle in endothelial growth medium-2 me-

ium. After 20 h of incubation, cells were harvested and counted. Then, 2 × 10^4 living cells were incubated at 37°C for 2 h in wells

precoated with vitronectin or fibronectin. Attached cells were stained

with crystal violet, and the incorporated dye was measured by reading

absorbance at 560 nm using a scanning multwell spectropho-

tometer.

Assessment of Endothelial Cell Viability and Proliferation. HUVECs were seeded at a density of 10^4 cells/well in 96-well micro-

titer plates precoated with 0.2% (v/v) gelatin (Sigma, Bornem, Bel-

gium). Twenty-four hours later, medium was removed and replaced

by 100 μl of basal medium (MCDB 131; Invitrogen) supplemented or not with 20% fetal bovine serum (proliferation or viability, respec-

tively) in the presence of the drugs to be investigated (10 μM final

concentration) or vehicle alone. Viability of HUVECs was deter-

mined by a colorimetric assay based on formazan dye formation

(WST-1; Boehringer Mannheim, Mannheim, Germany), which directly correlates with the number of metabolically active cells in the

culture. After incubation of the cells for a period of 20 h, 10 μl of

WST-1/well were added and further incubated for 2 h at 37°C. A
decrease in the number of viable cells results in a decrease in the

overall activity of mitochondrial dehydrogenases in the sample with

an ensuing loss in formazan dye formation. The formazan dye was

prepared as follows: a solution of Triton X-100 (20 mg/ml, Collagen

R; Serva, Heidelberg, Germany) with 1 volume of 10 times concentrated DMEM (Invitrogen, Paisley, Scotland), 1.5 volumes of sodium bicarbonate solution (15.6 mg/ml), and 0.1 volume of sodium hydroxide solution (1 M) to adjust the pH to 7.4. Collagen-embedded rat aortic rings were processed in cylin-
drical agarose wells and placed in triplicate in 60-mm bacteriologic

dishes containing 8 ml of serum-free MCDB-131 medium (Invitrogen) supplemented with 25 mM NaHCO3, 1% glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. These ex vivo organo-
typic cultures were treated with single compound concentrations ranging from 10 to 0.1 μM. After 9 days of culture at 37°C under a

air-CO2 (95%/5%) atmosphere, the aortic rings were photographed

under an optic microscope (25× magnification, Carl Zeiss AxioCam

HR Workstation, KS100 3.0 software). Images were digitalized into

1300 × 1300 pixels with 256 gray levels and assessed infographically

using Pesion 1.62 software. Areas of neovascularization and maximal

neofomed vessel lengths were evaluated as parameters of the ob-

served angiogenic response.

Statistical Analysis. All results are expressed as means ± S.D. Statistical significance was determined using the Student’s t test. Probability values less than 0.05 were considered to be significant.

Results

Original Torasemide Derivatives Bind to Human Platelet TXA2 Receptors. The evaluation of TP receptor affinity was performed by testing the ability of SQ-29,548, torasemide, and each original compound to displace [5,6-

3H]SQ-29,548, a strong competitive ligand of TP receptors, from its binding site on washed human platelets. Figure 1 shows that torasemide (IC50 = 2690 ± 72 nM) possessed a weak TP receptor affinity compared with SQ-29,548 (IC50 = 2.1 ± 2.0 nM). Results obtained with our original compounds demonstrated that each compound possessed a significantly enhanced receptor affinity, with BM-567 and BM-573 showing the highest affinities (IC50 = 1.1 ± 0.1 and 1.3 ± 0.1 nM, respectively).

Original Torasemide Derivatives Prevent U-46,619-

Induced Human Platelet Aggregation. The ability of our original compounds to prevent human platelet aggregation was determined using U-46,619, a TXA2 stable agonist, as inducer. The use of U-46,619 allowed us to estimate the IC50

each for original compound (Table 1). In this test, all of the
torasemide-derived compounds investigated displayed signific-
antly enhanced antiaggregatory properties compared with torasemide or furegrelate whereas SQ-29,548 exhibited a

Table 1

Drug concentrations required to fully prevent platelet aggregation induced by 1 μM U-46,619 and percentage of inhibition of platelet thromboxane synthase activity at concentrations of 100 and 10 μM

<table>
<thead>
<tr>
<th>Drugs</th>
<th>U-46,619 IC50</th>
<th>Thromboxane Synthase Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Torasemide</td>
<td>&gt;100</td>
<td>20.40 ± 1.58</td>
</tr>
<tr>
<td>BM-500</td>
<td>9.5 ± 2.4</td>
<td>92.52 ± 0.54</td>
</tr>
<tr>
<td>BM-520</td>
<td>0.336 ± 0.029</td>
<td>90.03 ± 5.19</td>
</tr>
<tr>
<td>BM-531</td>
<td>0.48 ± 0.09</td>
<td>99.89 ± 0.15</td>
</tr>
<tr>
<td>BM-567</td>
<td>0.298 ± 0.024</td>
<td>90.1 ± 3.31</td>
</tr>
<tr>
<td>BM-573</td>
<td>0.240 ± 0.013</td>
<td>93.40 ± 1.06</td>
</tr>
<tr>
<td>BM-600</td>
<td>0.421 ± 0.031</td>
<td>87.65 ± 3.17</td>
</tr>
<tr>
<td>BM-616</td>
<td>0.670 ± 0.142</td>
<td>88.61 ± 1.94</td>
</tr>
<tr>
<td>BM-519</td>
<td>0.664 ± 0.07</td>
<td>95.53 ± 0.36</td>
</tr>
<tr>
<td>BM-615</td>
<td>0.151 ± 0.012</td>
<td>85.14 ± 5.28</td>
</tr>
<tr>
<td>Furegrelate</td>
<td>&gt;100</td>
<td>89.90 ± 0.45</td>
</tr>
<tr>
<td>SQ-29,548</td>
<td>0.035 ± 0.007</td>
<td>12.30 ± 0.61</td>
</tr>
</tbody>
</table>

% inhibition 10 μM 100 μM
significantly higher preventive effect with an IC\(_{50}\) of 0.035 ± 0.007 \(\mu\)M. Among the torasemide derivatives tested, the highest antiaggregatory effects were observed with BM-567 and BM-573, which prevented U-46,619-induced platelet aggregation with IC\(_{50}\) values of 0.298 ± 0.042 and 0.240 ± 0.013 \(\mu\)M, respectively.

**Original Torasemide Derivatives Inhibit TXS Activity.** To determine whether our compounds displayed a dual TXA\(_2\) modulation activity, we evaluated their ability to decrease platelet TXA\(_2\) release occurring during platelet aggregation. This hypothesis was tested by measuring the levels of TXB\(_2\), a stable TXA\(_2\) metabolite, after platelet aggregation induced by AA. An important release of TXB\(_2\) was observed during AA-induced platelet aggregation (632 ± 179 versus 85,525 ± 11,481 pg/ml in PRP before and after treatment with AA, respectively). Pretreatment of platelets with our original torasemide derivatives at the final concentrations of 100 and 10 \(\mu\)M resulted in a significant and concentration-dependent inhibition of TXS activity (Table 1). Using the same assay, SQ-29,548, a TP receptor antagonist, failed to prevent this release whereas furegrelate, a TXS inhibitor, dose dependently inhibited TXS activity.

**Original Torasemide Derivatives Do Not Inhibit COX-1 and COX-2 Activities.** To assess the specificity of the TXS inhibitory profile of the drugs, we evaluated whether they were able to inhibit, at the final concentration of 10 \(\mu\)M, COX-1 and COX-2 using purified enzymes. Addition of AA (10 \(\mu\)M final concentration) to purified ovine COX-1 or COX-2 enzymes and further incubation for 2 min at 37°C led to the biosynthesis of quantifiable amounts (744 ± 245 and 10,237 ± 325 pg/ml, respectively) of PGE\(_2\). Preincubation with our original compounds or with torasemide for 60 min did not significantly modify PGE\(_2\) biosynthesis compared with controls (data not shown). Using the same experimental

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**Fig. 2.** Migration of HUVECs after treatment with various drugs using modified Boyden chambers. A, HUVECs were treated with U-46,619 at various concentrations or with 10 ng/ml VEGF, and the percentage of migrated cells in the different treatment conditions was compared with that of the control condition (vehicle). B, HUVECs were treated with torasemide, original torasemide-derived compounds, SQ-29,548, or furegrelate at final concentrations of 10 and 1 \(\mu\)M. Chemotaxis experiments were conducted using 10 ng/ml VEGF as chemoattractant. The percentage of migration inhibition was obtained using the following equation:

\[ \% \text{inhibition} = 1 - \frac{\text{number of migrated cells in the presence of VEGF and investigated drug}}{\text{number of migrated cells in the presence of VEGF}} \times 100. \]

\(+\), \(p < 0.05\); \(+ +\), \(p < 0.01\) (Student's \(t\) test).
conditions, indomethacin, a nonselective COX inhibitor, decreased PGE2 production with IC50 values of 0.104 and 0.5 
\mu M against COX-1 and COX-2, respectively.

**Original Torasemide Derivatives Inhibit Endothelial Cell Migration.** Migration of HUVECs was evaluated in 
Boyden chambers. The chemotactism of HUVECs was significantly enhanced by VEGF (10 ng/ml final concentration) and 
U-46,619 (0.3 and 0.1 
\mu M final concentrations) (Fig. 2A). Reference drugs and our original compounds were assayed for their ability to affect the migration of VEGF-challenged HUVECs through the chambers. As shown in Fig. 2B, all the 
drugs tested, except torasemide and BM-500, significantly reduced the migration of VEGF-challenged HUVECs at the 
concentration of 10 
\mu M. When the drugs were used at 1 
\mu M final concentration, migration inhibition remained statistically significant for SQ-29,548 and the torasemide deriv-
atives BM-519, BM-567, and BM-573.

**Original Torasemide Derivatives Do Not Affect the Viability and Adhesion of Endothelial Cells.** Incubation 
of HUVECs with our original compounds at final concentrations of 10, 1, and 0.1 
\mu M in the presence or absence of fetal bovine serum did not significantly inhibit formazan dye for-
mation compared with appropriate controls (data not shown). These data demonstrated the lack of cytotoxicity or antipro-
liferative effects of our original compounds on HUVECs. The impact of the torasemide-derived compounds on cellular ad-
hesion were also evaluated in cell adhesion assays using vitronectin- and fibronectin-coated microtiter plates. Neither 
U-46,619 (at final concentrations of 1, 0.3, and 0.1 
\mu M) nor the original compounds altered the adhesion of HUVECs to vitronectin or fibronectin (Fig. 3, A and B).

**Original Torasemide Derivatives Inhibit Intracellular Calcium Pool Mobilization in Endothelial Cells.** To
gain insight into the mechanisms by which our torasemide derivatives may inhibit endothelial cell migratory properties, we undertook to investigate whether these compounds may modulate the mobilization of intracellular Ca2+ pools in HUVECs. Several studies have indeed suggested the involve-
ment of intracellular Ca2+ fluxes in endothelial cell migra-
tion (Shukla et al., 2001; Joo et al., 2002; LaMontagne et al., 2006), and it has been shown previously that activation of 
TXA2 signaling leads to a release of Ca2+ from intracellular stores (Kinsella, 2001). We observed that U-46,619 (1 
\mu M final concentration) induced Ca2+ influxes in HUVECs with a measured intracellular Ca2+ mobilization of 3213 ± 300 nM. Pretreatment of HUVECs with BM-567 and BM-573 (10 
\mu M final concentration) significantly prevented U-46,619-
induced Ca2+ mobilization (1484 ± 49.5 and 1056 ± 108; \( P < 0.01 \) for each experimental condition).

**Torasemide-Derived Compounds Inhibit Angiogenesis in the ex Vivo Aortic Ring Assay.** Treatment of 
rat aortic rings with VEGF (20 ng/ml) resulted in a significant increase in vessel sprouting (Fig. 4A), which was 
quantified by measurement of the neovascularization area and the maximal vessel outgrowth length (0.937 ± 0.055 mm2 
and 1.44 ± 0.105 mm for 20 ng/ml VEGF-treated explants versus 0.630 ± 0.036 mm2 and 0.78 ± 0.301 mm 
for controls, respectively). U-46,619 at final concentrations of 0.3 and 0.1 
\mu M also significantly increased the neovas-
cularization area of the rat aortic ring explants whereas it 
decreased this neovascularization at the final concentra-
tion of 1 
\mu M (Fig. 4, B and C). SQ-29,548 and our original 
compounds were tested at final concentrations of 0.1, 1, 
and 10 
\mu M. Because they exhibited a weak inhibitory profile in the chemotaxis assay, BM-600 and BM-615 were 
not assayed. Experiments carried out with SQ-29,548 and

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**Fig. 3.** Adhesion to vitronectin (A) and fibronectin (B) after treatment with torasemide-derived compounds at 
the final concentration of 10 
\mu M, as assessed using a colorimetric assay based on crystal violet uptake by 
HUVECs, which directly correlates with the number of adhesive cells in the culture well.
torasemide showed that these drugs did not modify the angiogenic response of the rat aortic ring explants in terms of both neovascularization area and maximal vessel outgrowth length. Results obtained with our original compounds are summarized in Fig. 5, A and B. BM-616 significantly decreased the neovascularization area (Fig. 5, C, D and E). The torasemide derivatives were also investigated in this model under VEGF stimulation (20 ng/ml). Under these conditions, BM-519, BM-520, and BM-616 still significantly decreased the angiogenic response when evaluated in the presence of VEGF (Fig. 6, A and B).

**Discussion**

Dual TXA₂ receptor antagonists and TXS inhibitors have been described previously, including a family of agents derived from the TXS inhibitor isbogrel (Dogne et al., 2000). Ridogrel and terbogrel belong to this family. They exhibit inhibitory activities toward TXS and/or TP receptors that are weaker than or equivalent to those observed with our torasemide-derived compounds. They have been tested in clinical trials as antithrombotic and anti-inflammatory agents (Dogne et al., 2000; Carty et al., 2001). Yet, to the best of our
Fig. 5. Effect of torasemide and original torasemide derivatives on rat aortic explant vessel sprouting and area of neovascularization. Bars represent the percentage of capillary length (A) and area (B) reduction after treatment with the various drugs at final concentrations of 1 and 10 μM, compared with the appropriate controls. *p < 0.05; **p < 0.01 (Student’s t test). Representative photomicrographs of a control rat aortic ring explant (C) and rat aortic ring explants treated with BM-616 at final concentrations of 10 μM (D) and 1 μM (E).

Fig. 6. Evaluation of the effect of original torasemide derivatives in the rat aortic ring angiogenesis model under VEGF stimulation. The angiogenic response was quantified by the measurement of capillary areas and maximal capillary lengths, as detailed under Materials and Methods. Values are shown as means ± S.D. BM-616, BM-520, and BM-519 were tested at final concentrations of 0.1, 1, and 10 μM in the presence of 20 ng/ml VEGF. Bars represent the percentage of capillary length (A) and area (B) reduction after treatment with the various drugs at final concentrations of 0.1, 1, and 10 μM, compared with the appropriate controls. *p < 0.05; **p < 0.01 (Student’s t test).
knowledge, none of these molecules has been tested for its capacity to inhibit angiogenesis.

In this study, we have synthesized, produced, characterized, and selected nine compounds that modulate the pathway of TXA₂, a prostanoid involved in cancer development/progression. These original compounds, derived from torasemide, represent a new family of chemical molecules that act as TXA₂ receptor antagonists and TXA₂ synthase inhibitors and are able to inhibit endothelial cell migration in vitro and angiogenesis in an ex vivo animal model.

The present evaluation of our dual TXA₂ modulators demonstrates that these compounds, when used at the final concentration of 10 μM (with the exception of BM-500), significantly inhibit the migration of VEGF-challenged HUVECs. This inhibitory activity remains significant for BM-567, BM-573, and BM-519 when they are added to the cells at the final concentration of 1 μM. It is interesting to note that these three compounds, which have been identified as the most potent ligands of human platelet TP receptors, also appear to be the most efficient for preventing human platelet aggregation induced by U-46,619. This observation suggests a direct correlation between the potency of the compounds to modulate the TXA₂ pathway and their ability to inhibit VEGF-driven migration of endothelial cells.

In this study, we have further investigated the effects of our original torasemide derivatives in the rat aortic ring assay, which represents a valuable ex vivo angiogenic model because it does not require exogenous growth factors and recapitulates virtually all of the biologic steps of a complete and spontaneous angiogenic phenomenon. We have found that all torasemide-derived compounds assayed in this ex vivo angiogenesis model display significant antiangiogenic effects, with the highest inhibition obtained with BM-616. It is noteworthy that this molecule was not the most potent inhibitor of endothelial cell migration. This difference may, at least partly, be explained by the difference in stability of the different drugs tested. For example, BM-616 presents a particular substitution pattern with a bromine atom adjacent to the methyl group of R₂. This substitution is known to prevent the commonly observed oxidations of methyl substituents in the ortho position.

In humans, TXA₂ signals through two TP receptor isoforms named TPα and TPβ. These two TP receptors contain seven-transmembrane domains and can be coupled to a large number of G-proteins. Depending on the G-protein, various intracellular signaling pathways involving adenyl cyclase, phospholipase C, or AG1478-sensitive epidermal growth factor receptor, can be switched on (Sinead et al., 2002). Yet, TP receptors are thought to prominently signal through the activation of the β-isoenzymes of phospholipase C, leading to phosphatidylinositol turnover and release of Ca²⁺ from intracellular stores (Kinsella, 2001). Interestingly, several studies have suggested the involvement of intracellular Ca²⁺ fluxes in endothelial cell migration (Shukla et al., 2001; Joo et al., 2002; LaMontagne et al., 2006). In this context, we have sought to determine whether some of our most potent torasemide derivatives (BM-567 and BM-573) may in fact modulate the mobilization of intracellular Ca²⁺ pools in HUVECs. We have observed that BM-567 and BM-573 are able to decrease U-46,619-induced intracellular Ca²⁺ influxes in endothelial cells. Therefore, the ability of our drugs to inhibit endothelial cell migration may be explained, at least in part, by their interference with intracellular Ca²⁺ mobilization in HUVECs. These findings are in agreement with the general view that 1) the TXA₂ pathway signals through the activation of phospholipase C, leading to phosphatidylinositol turnover, and subsequent release of Ca²⁺ from intracellular stores (Kinsella, 2001) and 2) intracellular Ca²⁺ fluxes are involved in endothelial cell migration (Shukla et al., 2001; Joo et al., 2002; LaMontagne et al., 2006).

Accumulating evidence suggests an important role for the TXA₂ pathway in tumor growth and associated angiogenesis. For example, Pradono et al. (2002) have transduced a retroviral vector carrying TXS or prostacyclin synthase cDNA to colon-26 adenocarcinoma cells and have subsequently inoculated the transformants to BALB/c mice. They have observed that tumors derived from TXS transformants grow significantly faster than null-vector controls and show more abundant vasculature, indicating that the TXA₂ pathway may be a critical determinant for angiogenesis and subsequent tumor development. In the same model, tumors derived from prostacyclin synthase transformants grow significantly less than null-vector controls and show reduced vasculature. These data further illustrate the antagonistic effects of TXA₂ and prostacyclin (PGL₁) in tumor angiogenesis and clearly suggest that the selective targeting of COX-2 downstream mediators, such as TXA₂, may be an interesting approach to inhibit tumor-associated angiogenesis (Pradono et al., 2002).

In general terms, the growth of primary cancers is dependent on the angiogenic process, and this is also the case for metastatic lesions. Whether these lesions would respond similarly to anti-TXA₂ modulators still remains to be answered. Clinically, it is well known that some cancer types, such as clear cell carcinoma of the kidney, appear more “angiogenic” than others. This type of malignant lesion may therefore be more susceptible to an inhibition of the TXA₂ pathway. However, this theory undoubtedly remains to be tested in trials. Our findings describing the antiangiogenic effects of dual TXA₂ modulators might be useful in other therapeutic areas. Indeed, angiogenesis is also a critical process and a potential therapeutic target in several nonmalignant diseases such as rheumatoid arthritis and osteoarthritis (Walsh, 1999), diabetic retinopathy (Gargiulo et al., 2004), psoriasis (Leong et al., 2005), endometriosis (Fujimoto et al., 1999), and pre-eclampsia (Ahmad and Ahmed, 2004).

The key role of prostanoids in cancer is unanimously recognized and has been demonstrated through epidemiologic, experimental, and clinical observations. It is also generally admitted that the toxicity associated with the administration of COX inhibitors, whether selective or not, limits their use in cancer prevention strategies as well as for the treatment of patients suffering from chronic diseases. Indeed, uptake of NSAIDs is commonly associated with gastrointestinal side effects. This toxicity is related to the ability of these drugs to inhibit the COX-1 isoform, which participates in gastric mucosa protection through the synthesis of prostaglandins (de Leval et al., 2000). This gastrointestinal toxicity is not observed with COX-2 selective inhibitor treatment. However, rofecoxib, a selective COX-2 inhibitor, was withdrawn from the market at the end of September 2004 because of an increased risk of cardiovascular events. This cardiovascular toxicity has been demonstrated in two double-blind, randomized clinical trials: the APPROVe (Adenomatous Polyp Pre-
von VIOXX and VIGOR (Vioxx Gastrointestinal Outcomes) studies, which have demonstrated a 2-fold increase in the risk of serious thromboembolic events, including stroke and myocardial infarction in the rofecoxib-treated group compared with the placebo group (Bombrider et al., 2000; Bre-salier et al., 2005). This cardiovascular toxicity can be associated with the COX-2 inhibitory properties of rofecoxib. Indeed, although COX-2 is an inducible enzyme expressed in pathologic conditions such as inflammation and cancer progression, this enzyme is also expressed in physiologic conditions by several tissues such as vascular endothelium, kidney, and brain. In terms of cardiovascular physiology, COX-2 has been demonstrated to be the prominent source of PGL₂ in vivo (Belton et al., 2000). This prostanoid displays powerful vasodilatative and antiaggregatory properties. On the other hand, COX-1 and TXA₂ synthase are constitutively expressed within platelets. COX-1 is responsible for the enzymatic transformation of arachidonic acid into PGH₂ which is subsequently transformed by the TXA₂ synthase into TXA₂, a potent inducer of platelet aggregation and vasoconstriction (Dogne et al., 2004). Nonspecific NSAIDs block both COX isoforms and therefore have a balanced effect on reducing the prothrombotic actions of TXA₂ and the antithrombotic effects of PGI₂ (Dogne et al., 2004). In contrast, rofecoxib suppresses the formation of endogenous PGI₂ without affecting TXA₂ generation in healthy volunteers, potentially creating an alteration of the vascular homeostasis (FitzGerald, 2003). Such results have also been demonstrated with celecoxib, another COX-2 selective inhibitor. The shift in the TXA₂/PGI₂ balance is a strong theoretical basis for an association between use of coxibs and the occurrence of thromboembolic disorders (Pratico and Dogne, 2005). In this context, our original dual TXA₂ modulators should have a positive effect on the TXA₂/PGI₂ balance and may prevent thromboembolic occurrence in vivo. Our original compounds indeed combine the advantages of pure TXA₂ receptor antagonist and TXS inhibitors: blocking the agonistic activities of TXA₂ on its receptors and allowing the accumulation of endoperoxide PGH₂ that can be metabolized to PGI₂ by endothelial cell prostacyclin synthase (Marcus et al., 1981). It is also noteworthy that our original compounds lack inhibitory effects against COX-1 or COX-2 activity in vitro. Therefore, we think that no major prothrombotic adverse effect should be expected from the administration of our compounds. Yet, we acknowledge that this speculation, based on strong in vitro data, should be confirmed. Overall, we believe that our toraseide derivatives represent an exciting and potentially viable alternative to COX inhibitors for the prevention and treatment of diseases in which angiogenesis is turned on.

References


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