Terutroban, a Thromboxane/Prostaglandin Endoperoxide Receptor Antagonist, Increases Survival in Stroke-Prone Rats by Preventing Systemic Inflammation and Endothelial Dysfunction: Comparison with Aspirin and Rosuvastatin

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ABSTRACT

This study investigated the efficacy of terutroban, a specific thromboxane/prostaglandin endoperoxide receptor antagonist, on stroke incidence in spontaneously hypertensive stroke-prone rats (SHRSP). The effects of terutroban were compared with those of aspirin, another antiplatelet agent, and rosuvastatin, known to exert end-organ protection in SHRSP. Salt-loaded male SHRSP were treated orally once a day with vehicle, terutroban (30 mg/kg/day), aspirin (60 mg/kg/day), or rosuvastatin (10 mg/kg/day). Compared with vehicle, and regardless of any effect on blood pressure or serum thromboxane B2 levels, terutroban significantly increased survival (p < 0.001) as a consequence of a delayed brain lesion occurrence monitored by magnetic resonance imaging (p < 0.001), and a delayed increase of proteinuria (p < 0.001). Terutroban decreased cerebral mRNA transcription of interleukin-1β, transforming growth factor-β, and monocyte chemoattractant protein-1 after 6 weeks of dietary treatment. Terutroban also prevented the accumulation of urinary acute-phase proteins at high molecular weight, identified as markers of systemic inflammation, and assessed longitudinally by one-dimensional electrophoresis. Terutroban also has protective effects on the vasculature as suggested by the preservation of endothelial function and endothelial nitric-oxide synthase expression in isolated carotid arteries. These effects are similar to those obtained with rosuvastatin, and superior to those of aspirin. Terutroban increases survival in SHRSP by reducing systemic inflammation as well as preserving endothelial function. These data support clinical development of terutroban in the prevention of cerebrovascular and cardiovascular complications of atherothrombosis.

Several clinical and experimental studies (Widlansky et al., 2003; Huang and Vita, 2006) support the hypothesis that endothelial dysfunction and systemic inflammation play key roles in the pathogenesis of vascular diseases, including myocardial and brain ischemia. Human studies have demonstrated positive association between systemic inflammation induced by endotoxin infusion and marked endothelial dysfunction as well as impaired responses to vasoactive compounds (Plieger et al., 2004). An analysis of the Framingham Heart Study Offspring cohort found that serum C-reactive protein, IL-6, and soluble intercellular adhesion molecule-1 levels inversely correlated with brachial artery flow-mediated dilation and reactive hyperemia in the forearm, although this relationship was weakened after adjusting for traditional risk factors (Vita et al., 2004).

Spontaneously hypertensive stroke-prone rats (SHRSP) develop hypertension and proteinuria and die after the onset of this work was supported by Institut de Recherches Internationales Servier, France (Protocol Code PHA-18886-033-ITA). V.B.-G. and L.L. are employees of Servier.

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ABBREVIATIONS: IL, interleukin; SHRSP, spontaneously hypertensive stroke-prone rats; HMW, high molecular weight; TP, thromboxane/prostaglandin endoperoxide; TP, thromboxane/prostaglandin endoperoxide receptor; ASA, aspirin; RSV, rosuvastatin; S 18886, 3-((6-amino-(4-chlorobenzenesulfonyl)-2-methyl-5,6,7,8-tetrahydronaphthalen-1-yl)propionic acid; MRI, magnetic resonance imaging; LMW, low molecular weight; TXB2, thromboxane B2; RT-PCR, reverse transcription-polymerase chain reaction; TGF, transforming growth factor; MCP, monocyte chemoattractant protein; eNOS, endothelial nitric-oxide synthase; L-Phe, L-phenylephrine; Ach, acetylcholine; NAME, N’-nitro-L-arginine methyl ester; ANOVA, analysis of variance; pD2, sensitivity; BayU3405, ramatroban; ONO-8809, n-decyl (3-(4-bromobenzenesulfonylaminomethyl)(bicyclo[2.2.1]hept-2-yl)-5-hexanoate; PG, prostaglandin.
of cerebrovascular damage, which is invariably preceded by systemic inflammation and endothelial dysfunction (Sironi et al., 2001; Ballerio et al., 2007). It is noteworthy that systemic inflammation is characterized by an accumulation—in serum and urine—of acute-phase high-molecular-weight (HMW) proteins, such as thiostatin, the most common marker of inflammation in the rat (Sironi et al., 2001). In SHRSP, brain lesions have a vasogenic origin due to the blood-brain barrier impairment (Guerrini et al., 2002). Therefore, this model is particularly suited to reveal cerebrovascular benefits of drugs acting on the inflammatory cascade and/or endothelial dysfunction.

The purpose of this study was to evaluate, in SHRSP, the effects of terutroban, a highly selective and long-acting thromboxane/prostaglandin endoperoxide (TP) receptor (TPr) antagonist with antithrombotic, antivasoconstrictive, and anti-inflammatory/antiatherosclerotic properties (Cimetièr et al., 1998). Previous experimental studies have demonstrated that terutroban prevents vascular wall proliferation and atherogenesis (Cheng et al., 2002; Viles-Gonzalez et al., 2005; Worth et al., 2005); increases antioxidant enzymes, such as glutathione peroxidase (Sebekova ´ et al., 2007); and has anti-inflammatory actions in vitro and in vivo (e.g., decreased macrophage infiltration and intercellular adhesion molecule-1; Cayatte et al., 2000). Terutroban also improved endothelial function in patients with coronary artery disease treated with aspirin (Bellantoni et al., 1998). Terutroban is developed in secondary prevention trials in patients with a history of ischemic stroke or transient ischemic attack (Bousser et al., 2009a,b).

In this study, the optimally effective dose of terutroban established in previous work was compared with those of aspirin (ASA) and rosuvastatin (RSV) to provide comparative data on end-organ protection and anti-inflammation in SHRSP (Sironi et al., 2005).

## Materials and Methods

### Animals and Protocol

Male SHRSP aged 4 to 5 weeks were obtained from Charles River Italia (Calco, Italy) and were cared for in accordance with our institution’s guidelines. Fifty-two SHRSP were housed in metabolic cages for 24 h to collect urine for proteinuria determinations by Bradford method (Bradford, 1976) and proteomic studies. Blood was drawn every week from the tail vein; serum was obtained and stored at −20°C until analyzed. All rats underwent weekly magnetic resonance imaging (MRI) until 24-h proteinuria reached 100 mg/day, and then every 2 days until cerebrovascular damage was detected. After 6 weeks (i.e., when the vehicle-treated rats developed brain lesions), five animals from each group were sacrificed to collect the brain as well as the carotid artery.

### MRI Evaluation of Brain Damage

The rats were anesthetized with 1.5% isoflurane (Merial, Toulouse, France) in 70% N₂, 30% O₂, and then they were placed inside a Avance II 4.7T spectrometer (Bruker, Newark, DE) with a microimaging accessory. After a scout image, 16 contiguous 1-mm-thick slices were analyzed caudally to the olfactory bulb using a field of view of 4 × 4 cm², a turbo spin echo sequence with 16 echoes per excitation, 10-ms interecho time, 85-ms equivalent echo time, and 4-s repetition time. Eight T2-weighted images of 128 × 128 pixels (zero-filled to 256 × 256) were averaged

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**Fig. 1.** Effects of vehicle (squares; n = 8), ASA (triangles; n = 8), RSV (stars; n = 8), and terutroban (circles; n = 8) on systolic blood pressure (A), appearance of brain damage (B), and survival (C). In B, ***p < 0.001; ***, p < 0.05 versus vehicle; and #, p < 0.05 versus terutroban and RSV. In C, *, p < 0.05 and ***, p < 0.001 versus vehicle group. D, representative MRI images from healthy (left) and damaged (right) brain; the lesion(s) visualized by T2W-MRI appears as a hyperintense area, pointed out by the arrows.
in 8 min 30 s. The occurrence of lesions was defined as the presence of areas of high signal intensity on T2-weighted images.

**Proteinuria Studies.** One-dimensional electrophoresis of urine proteins (50 µg) was run in the presence of SDS without sample reduction in a discontinuous buffer system on 4 to 12% polyacrylamide gels stained with colloidal blue. Densitometry was performed using Quantity One version 4.5.2 (Bio-Rad Laboratories, Hercules, CA) to evaluate the percentage of low-molecular-weight (LMW) and HMW proteins density.

**Determination of TXB₂ and 11-Dehydro-TXB₂.** The serum levels of TXB₂, and urinary levels of 11-dehydro-TXB₂, were measured using commercial kits (Cayman Chemical, Ann Arbor, MI).

**Brain Tissue Expression of Inflammatory Markers.** After 6 weeks of dietary treatment, five animals from each group were sacrificed to collect the brains. Total RNA was prepared by means of guanidium thiocyanate denaturation from forebrain homogenates. Reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate the expression of IL-1β, TGF-β, and MCP-1. All of the reagents were used were purchased from Invitrogen (Carlsbad, CA).

**Expression of eNOS.** eNOS expression was evaluated by RT-PCR on carotid artery homogenates from animals sacrificed after 6 weeks of treatment.

**Endothelial Dysfunction.** Isolated carotid artery rings (3 mm) were suspended in an individual organ bath filled with Krebs’ solution, and their vascular reactivity was evaluated as described previously (Ballario et al., 2007). Indomethacin (10⁻⁵ M; Chiesi Farmaceutici S.p.A., Parma, Italy) was added to Krebs’ solution to inhibit prostanoi synthesis. Arteries were challenged with KCl (100 mM) to check the viability of tissues; vessels not responding to KCl were discarded. Vascular smooth muscle function was determined by cumulative addition of 10⁻⁹ to 10⁻⁵ M l-phenylephrine (l-Phe; Sigma-Aldrich), the contraction response being expressed as the percentage of KCl response. Subsequently, the rings were constricted to their individual EC₅₀ value for l-Phe, and maximal smooth muscle relaxation to 10⁻¹⁶ to 3 x 10⁻⁹ M sodium nitroprusside was determined (Sigma-Aldrich). After washout, the rings were constricted to their individual EC₅₀ value for l-Phe, and endothelium-dependent relaxation in response to 10⁻⁹ to 10⁻⁵ M acetylcholine (Ach; Sigma-Aldrich) was studied both in the absence and presence of 10⁻⁴ M l-NAME. The relaxation responses were expressed as the percentage of l-Phe-induced contraction.

**Statistics.** Between-group differences were computed by analysis of variance (ANOVA) followed by an appropriate post hoc test; the between-group differences in proteinuria and LMW and HMW protein density were computed by ANOVA for repeated measurements over time followed by Tukey’s post hoc test. An unpaired t test was used to compare baseline and vehicle-treated group data. Concentration-response curves were statistically analyzed using ANOVA followed by Tukey’s or Tamhane’s T2 post hoc test. Sensitivity to the antagonists (pD₂) was expressed as the negative logarithm of half-maximal effective concentration (EC₅₀) calculated from individual curves. Results are expressed as means ± S.D. p < 0.05 was considered statistically significant.

**Results**

**Physiological Parameters and Survival of SHRSP.** Body weight increased similarly in all experimental groups. The severe hypertension that developed was not affected by any of the drug treatments (Fig. 1A). Plasma total cholesterol and triglyceride levels (42.38 ± 3.89 and 68.5 ± 8.96 mg/dl, respectively, at baseline) did not significantly change during the treatment period in any groups. Vehicle-treated animals developed cerebral lesions 42.4 ± 10.8 days after starting salt loading. All treatments significantly delayed the appearance of cerebrovascular damage (Fig. 1, B and D). However, the delay of occurrence observed under terutroban (87.6 ± 19.2 days; p < 0.001) was greater than that induced by aspirin (63.9 ± 9.01 days; p < 0.05) and comparable with that observed after RSV (85.6 ± 16.9 days; p < 0.001). Comparison of survival clearly shows the effectiveness of all treatments (Fig. 1C). Compared with the vehicle group, survival was similarly increased by terutroban and RSV (p < 0.001), and this effect was significantly superior to that observed after aspirin treatment (RSV, p < 0.05; terutroban, p < 0.01).

**Serum TXB₂ and Urinary 11-Dehydro-TXB₂ Levels.** As expected, serum TXB₂ and urinary 11-dehydro-TXB₂ levels were significantly decreased by ASA, whereas the levels were not affected by salt loading, RSV treatment, or terutroban treatment (Fig. 2, A and B).

**Proteinuria and Composition of Urinary Proteins.** The SHRSP receiving vehicle developed progressively a severe proteinuria. After 4.7 ± 1.3 weeks of salt loading, proteinuria was higher than 100 mg/day and increased rapidly and linearly to reach an average of 266 ± 28.9 mg/day after 7 weeks. Treatment with terutroban and RSV delayed significantly the increase in proteinuria (10.5 ± 3.4 weeks, p < 0.001 and 9.1 ± 1.9 weeks, p < 0.01 versus vehicle, respectively) (Fig. 2C), whereas ASA had only a slight effect (6.2 ± 0.05 weeks; p < 0.01 versus vehicle, terutroban, and RSV; and #, p < 0.05 versus vehicle; and ##, p < 0.01 versus terutroban.

**Fig. 2. A and B, effects of vehicle, ASA, RSV, and terutroban treatment on serum TXB₂ and urinary 11-dehydro-TXB₂ levels (n = 5/group); ***, p < 0.001 versus vehicle, terutroban, and RSV; and #, p < 0.05 versus vehicle and RSV. C, delay in the appearance of proteinuria >100 mg/day; ***, p < 0.001; **, p < 0.01 versus vehicle; and ##, p < 0.01 versus terutroban.
1.2 weeks, N.S.) on this parameter. At the beginning of the experiment (see week 1 in Fig. 3), the most abundant excreted protein was the major urinary protein (α2u-globulin), which represents the major protein excreted in urine of healthy male rats (Sironi et al., 2001). Major urinary protein, together with other LMW proteins, accounted for approximately 70% of the total protein content. Protein composition changed over time in the salt-loaded SHRSP, with an accumulation of HMW proteins identified previously as markers of inflammation (Ballerio et al., 2007), and a simultaneous decrease in LMW proteins (Fig. 3A). In the vehicle group, HMW proteins reached 70% of the total protein content 4 to 5 weeks after the start of dietary treatment (Fig. 3A); in the ASA- and RSV-treated rats, HMW proteins became preponderant after 7 and 9 weeks, respectively (Fig. 3, B and C). Densitometric analysis of the excreted proteins in the terutroban group showed that the HMW protein level did not increase to more than 50% of total protein content even after 14 to 15 weeks of treatment (Fig. 3D).

Brain Expression of Inflammatory Markers. In comparison with vehicle-treated animals, all of the drugs markedly reduced the accumulation of IL-1β, MCP-1, and TGF-β mRNA in the brain tissues (Fig. 4).

Endothelial Dysfunction. The response curves to phenylephrine in carotid artery rings showed significantly reduced contraction in terutroban-treated rats (p < 0.05). ASA and RSV treatment also tended to reduce the contractions caused by phenylephrine (N.S.) (Fig. 5A). In rings precontracted with phenylephrine, the concentration-response curves to the administration of the NO donor sodium nitroprusside were comparable in all groups (Fig. 5B). The endothelium-dependent relaxation evoked by acetylcholine was not altered by ASA, but it was significantly increased by terutroban and RSV (p < 0.01) (Fig. 5C). Incubation with

Fig. 3. Analysis of urinary proteins by one-dimensional electrophoresis. Left, results of the densitometric analyses expressed as the percentages of HMW and LMW molecular weight proteins over time in rats treated with vehicle (A), ASA (B), RSV (C), or terutroban (D); n = 6/group. Right, representative images of gels for each condition. MW, molecular weight. *, p < 0.05 versus densitometric HMW value at week 1; and §, p < 0.05 versus densitometric LMW value at week 1.
Terutroban Increases Survival in Stroke-Prone Rats

Discussion

In this study, we investigated the effects of terutroban, a TP receptor antagonist, on the pathological events that spontaneously develop in SHRSP. The effects induced by terutroban were compared with those of aspirin, a cyclooxygenase inhibitor, and rosuvastatin, a statin that demonstrated beneficial effects in this model (Sironi et al., 2005). In salt-loaded SHRSP, terutroban delayed the occurrence of spontaneous brain lesions and consequently increased the survival, regardless of any effect on blood pressure or on serum TXB₂ levels. Terutroban was more effective in brain damage protection than aspirin, and it had similar effects as rosuvastatin. In a previous study, a beneficial effect was also observed when terutroban was administered 3 weeks after the start of dietary treatment, indicating that terutroban can also reverse ongoing pathological events in salt-loaded SHRSP (Gelosa et al., 2007).

Mechanisms that could contribute to the beneficial effect of TP receptor blockade on stroke prevention in this model are an attenuation of the systemic inflammation that invariably precedes the occurrence of cerebrovascular events, as well as a preservation of vascular reactivity.

One of the features of the systemic inflammation that develops in SHRSP is the progressive urinary accumulation of HMW proteins (Sironi et al., 2001), which reached 70% of total urinary protein excretion after 4 to 5 weeks of dietary treatment. Previously published data have shown that these proteins, solved with two-dimensional electrophoresis, consist of markers of inflammatory response, such as kallikrein-binding protein, transthyretin, albumin, α-1-antitrypsin, and thiostatin. These proteins are markers of an inflammatory response and their accumulation in body fluids invariably precedes the occurrence of brain abnormalities (Sironi et al., 2001). Terutroban significantly attenuated this accumulation with a level of HMW proteins less than 50%, confirming the major anti-inflammatory activity of terutroban at the systemic level, which is superior to that of rosuvastatin and aspirin treatments. Terutroban also prevented the accumulation of IL-1β, MCP-1, and TGF-β mRNA in brain tissue. These data are consistent with previous in vitro and in vivo experiments showing that TPr stimulation is significantly involved in inflammatory processes and that blockade of this receptor with terutroban reduced inflammatory markers in various experimental models (Cayatte et al., 2000; Zuccollo et al. 2005; Xu et al., 2006). Ishizuka et al. (2000) have also reported that TP receptor blockade with ramatroban (BayU3405) suppressed the expression of inflammatory mediators (particularly MCP-1) in stimulated vascular endothelial cells and that the TPr antagonist ONO-8809 contributed to cerebral protection in salt-loaded SHRSP by reducing macrophage accumulation and matrix metalloproteinase-9 activity (Ishizuka et al., 2007). Similarly to terutroban, rosuvastatin and aspirin significantly attenuated brain inflammation. We have reported previously that rosuvastatin prevented inflammatory processes associated with cerebrovascular disease, independently of changes in plasma lipid levels (Sironi et al., 2005). Aspirin had also demonstrated numerous pharmacological activities, including antioxidant and anti-inflammatory effects. Ishizuka et al. (2008) recently revealed that aspirin may inhibit the cerebrovascular inflammation in SHRSP through antioxidative properties.

In addition to its anti-inflammatory activity, terutroban significantly preserved vascular reactivity to a greater extent than aspirin and rosuvastatin. Analysis of the concentration-response curves of carotid artery rings showed that terutroban reduces the contraction elicited by phenylephrine, without affecting pD₂, indicating that the adrenergic receptor signal transduction mechanisms are not altered. Aspirin and rosuvastatin have beneficial but lesser effect on this parameter.

The endothelium-dependent relaxation induced by acetylcholine was significantly improved by terutroban and rosuvastatin in comparison with vehicle and aspirin. This result is consistent with clinical data showing that a single oral dose of terutroban significantly improved endothelium-dependent vasodilation in the peripheral arteries of patients with coronary artery disease treated with aspirin, thus strengthening the hypothesis that terutroban has additional therapeutic benefits, such as 1) allowing the
production of vasodilating prostanoioids (e.g., prostacyclin), which is impaired by cyclooxygenase inhibition; and 2) inhibiting the production of vasoconstrictor prostanoioids other than TXA2 (Cayatte et al., 2000). Moreover, the improvement of endotheliument-dependent relaxation was inhibited after incubation with L-NAME, suggesting a partial restoration of NO release or synthesis by terutroban. This hypothesis is strengthened by the increased expression of eNOS mRNA in the carotid arteries of animals treated with terutroban, whereas expression of eNOS mRNA was not changed significantly in animals treated with aspirin. This finding is in agreement with previous results showing an increase in eNOS expression in aorta of diabetic mice treated by terutroban (Zuccollo et al., 2005).

Benefits on survival induced by terutroban were independent of modifications in TXB2 levels, which remained unchanged after terutroban administration, contrary to aspirin, which almost suppressed the production of TXA2 (as reflected by a reduction of its serum metabolite TXB2) but with an effect on survival that was significantly inferior to that obtained with terutroban. The greater effect of TPr antagonism with terutroban on brain protection could be attributed to a greater effect in inflammation processes at the systemic level, and it is probably due to ligands other than TXA2 and prostaglandins-endoperoxides (PGG2 and PGH2). It was beyond the scope of our study to identify the eicosanoids potentially responsible for activating the inflammatory cascade involved in end-organ damage in SHRSP, but possible candidates are the isoprostanes, produced from arachidonic acid by nonenzymatic oxidation, and whose formation is not influenced by cyclooxygenase inhibitors. This hypothesis is corroborated by the results obtained by Ishizuka et al. (2007) who suggested that cerebrovascular inflammation in salt-loaded SHRSP may be due to TP receptor stimulation by 8-iso-PGF2α.

In this study, the effect of terutroban was similar to that of rosuvastatin, an effective drug in preventing end-organ damage in a model of SHRSP (Sironi et al., 2005). Terutroban increased survival to a greater extent than aspirin, probably due to its greater effects on systemic inflammation and endothelial dysfunction. The benefits of terutroban on survival and pathological events occurring in SHRSP may therefore be attributed to its anti-inflammatory activity, along with the improvement of endothelial function.

Controlling inflammation and preserving endothelial function are key factors for preventing the development of the spontaneous brain damage occurring in SHRSP. In addition to platelet aggregation inhibition, terutroban also offers the therapeutic benefit of anti-inflammatory and vascular protective properties, which support its clinical development in the prevention of cerebrovascular and cardiovascular complications of atherothrombosis.

Acknowledgments

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| Table 1 |

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Fig. 5. Effects of the in vivo pharmacological treatments on the cumulative concentration-response curves of carotid rings from SHRSP. A, phenylephrine-induced contraction: *, p < 0.05 terutroban versus vehicle group. B, sodium nitroprusside-induced relaxation. C, acetylcholine-induced relaxation before and after incubation with L-NAME (10^-4 M). **, p < 0.01 terutroban versus vehicle group; and $$, p < 0.01 RSV versus vehicle group. Data were collected from five rats for each experimental condition.


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