Selective Blockade of Endothelin-B Receptors Exacerbates Ischemic Brain Damage in the Rat

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Background and Purpose—Endothelins act through 2 receptors, namely, ET_A and ET_B. In the cerebral circulation, ET_A mediates marked and prolonged vasoconstriction, and its blockade increases cerebral blood flow (CBF) and reduces ischemic brain damage. However, the role of ET_B receptors remains unclear. In this study we examined, in rats, the kinetics of expression of ET_B and the effects of ET_B blockade on changes in CBF and brain damage after focal cerebral ischemia and N-methyl-D-aspartate (NMDA)–induced excitotoxic injury.

Methods—Rats were subjected to transient (60 minutes) focal cerebral ischemia or cortical injection of NMDA. The selective ET_A antagonist BQ-788 was injected intracerebroventricularly 30 minutes before and 30 minutes after the onset of ischemia. Cortical perfusion was monitored by laser-Doppler flowmetry. The volume of infarction or NMDA-induced cortical lesion was assessed at 24 hours after the insult. The reverse transcription–polymerase chain reaction technique was used to assess ET_B expression.

Results—Cerebral ischemia failed to alter the expression of ET_B mRNA in both acute and chronic stages. Administration of BQ-788 resulted in an increase in infarction volume (178%; P<0.05) accompanied by a decrease in residual CBF (−26.7% versus control; P<0.01). In these animals we found a positive correlation between the volume of infarction and the severity of the decrease in CBF. NMDA-induced cortical lesions were not affected by the administration of BQ-788.

Conclusions—Our results suggest that the ET_B antagonist BQ-788 induces deleterious effects that are mediated by the reduction of residual blood flow after ischemia and argue that the optimal therapeutic strategy in stroke would be to target the use of selective ET_A antagonists and not mixed ET_A/ET_B antagonists. (Stroke. 2002;33:3019-3025.)

Key Words: cerebral ischemia • endothelins • receptors, endothelin • vasoconstriction • rats

Endothelins are a family of 3 isopeptides, termed endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), recognized for their potent vasomotor activity.1,2 Two distinct types of endothelin receptors have been identified: ET_A and ET_B. The ET_A receptor is characterized by a higher affinity toward ET-1 than ET-2 and ET-3, while the ET_B receptor displays a similar affinity toward all 3 endothelins.3,4 Overall, the elements of the endothelin system (ET-1, ET-3, ET_A, ET_B, and endothelin-converting enzymes), but not ET-2, are expressed in both vascular and nonvascular compartments of the central nervous system.5,6 In the cerebral circulation, ET_A receptors mediate a potent and long-lasting vasoconstriction;7 however, the function of ET_B receptors is more controversial. As shown in many studies, the selective activation of ET_B receptors induces vasodilatation mediated by the generation of the endothelium-derived relaxing factor (nitric oxide and/or prostacyclin).8–10 Under some conditions ET_B can also induce vasoconstriction.10–12 This dual role of ET_B receptor, which is well described in some peripheral vessels in terms of pharmacological approaches, has led to a proposed subdivision of ET_B receptors into ET_B1 receptors (on endothelial cells) that mediate vasodilatation and ET_B2 receptors (on smooth muscle cells) that mediate vasoconstriction.5,13,14 However, no molecular correlates of these ET_B receptor subtypes have been reported.15 The demonstration of constrictive ET_A along with dilatory and/or constrictive ET_B receptors has important implications for the development of endothelin receptor antagonists with an optimal pharmacological profile (selective ET_A or mixed ET_A/ET_B) for the treatment of cerebrovascular diseases such as cerebral ischemia.16

Three lines of evidence point to a major role of ET-1 in the pathophysiology of cerebral ischemia: (1) the ability of exogenous ET-1 to reduce cerebral blood flow (CBF) to levels that induce infarction17; (2) the increase in plasma, cerebrospinal fluid, and cerebral tissue ET-1 levels after cerebral ischemia18,19; and (3) the efficacy of selective ET_A or mixed ET_A/ET_B antagonists in some, but not all, studies to reduce brain damage after experimental stroke.20–22 The precise mechanisms of ET-1 actions in cerebral ischemia...
have not yet been fully elucidated. The majority of studies that have intervened with endothelin receptor antagonists after experimental stroke have focused on the role of ET\textsubscript{B} receptors as mediators of brain damage. In contrast, the role of ET\textsubscript{A} receptors in ischemic pathology is still to be elucidated.

In the present study we focused on ET\textsubscript{B} receptors and addressed 3 questions: (1) the kinetics of ET\textsubscript{B} mRNA expression after cerebral ischemia; (2) the effects of selective ET\textsubscript{B} blockade on CBF changes and brain damage after ischemia; and (3) the effects of ET\textsubscript{B} blockade on N-methyl-D-aspartate (NMDA)–induced excitotoxic brain damage.

Materials and Methods

Anesthesia and General Preparation

The experiments were performed on adult male Sprague-Dawley rats weighing 347±9 g (R. Janvier Breeding Center) according to the appropriate European Directives and French National Legislation. Anesthesia was induced with halothane (5%) and maintained during surgery (0.7% to 1.3%) in an O\textsubscript{2}/N\textsubscript{2}O mixture (30%/70%). The animals were orally intubated and mechanically ventilated (Harvard Apparatus 683). Rectal temperature was recorded and kept close to 37.5°C with a heating pad (Thermalert). The caudal artery was cannulated with polyethylene tubing for continuous arterial pressure monitoring (Stoelting) as well as for gas analyses and pH (Ciba Corning).

Rats were assigned to the following experimental groups: (1) kinetics of expression of ET\textsubscript{B} mRNA after cerebral ischemia; (2) effects of BQ-788 on changes in CBF and brain damage after focal cerebral ischemia; and (3) effects of BQ-788 on cortical lesions induced by NMDA administration.

Middle Cerebral Artery Occlusion

Temporary focal cerebral ischemia was induced by occlusion of the right middle cerebral artery (MCAO) with the use of the intraluminal filament technique.\textsuperscript{23} Briefly, a nylon thread (0.18 mm in diameter) with a distal cylinder (3 mm in length and 0.38 mm in diameter) was inserted into the lumen of the external carotid artery and advanced to the origin of the MCA. The nylon thread was removed 60 minutes later to allow reperfusion. For sham-operated animals, the nylon thread was advanced to the origin of the MCA. The nylon thread was removed 60 minutes later to allow reperfusion. For sham-operated animals, the nylon thread was advanced to the origin of the MCA and immediately removed. The animals were then allowed to recover from anesthesia and were killed 24 hours after MCAO for histological analysis or at different time points for reverse transcription–polymerase chain reaction (RT-PCR) analyses.

Measurement of CBF

To examine the effects of the ET\textsubscript{B} antagonist (BQ-788) on cortical brain perfusion, rats were placed prone in a stereotaxic frame (Kopf Instruments). A laser-Doppler flowmetry (LDF) probe (0.7 mm in diameter, FioLab Moor Instruments) was positioned on the right hemisphere, at the time points analyzed, showed no change after MCAO compared with that observed in the respective contralateral hemisphere and with sham-operated animals. The housekeeping gene \( \beta \)-actin also showed no change over time (Figure 1). Similarly, the lesion induced by NMDA injection

Drug Characteristics and Administration

BQ-788 is a potent selective and competitive ET\textsubscript{B} receptor antagonist with an approximately 1000-fold relative selectivity for the ET\textsubscript{B} receptor.\textsuperscript{28} BQ-788 was purchased as a pure powder and dissolved in sterile dimethyl sulfoxide (DMSO) following the recommendations of the supplier (France-Biochem). The peptide nature of BQ-788 precluded its intravenous administration. BQ-788 or its vehicle was infused over 5 minutes into the right lateral ventricle (3 \mu L) (coordinates 0.8 mm posterior, 1.5 mm lateral, 4.3 mm ventral to the bregma)\textsuperscript{24} 30 minutes before and 30 minutes after the onset of MCAO or cortical administration of NMDA. NMDA was purchased as pure powder (Sigma-Aldrich) and dissolved in PBS buffer.

Reverse Transcription–Polymerase Chain Reaction

Brain tissue was collected 3 hours, 6 hours, 24 hours, or 3 days after transient MCAO. Total RNAs were isolated from the ipsilateral or contralateral hemispheres through the use of the RNaxel kit (Eurobio). Two micrograms of total RNAs was reverse transcribed into cDNA with the use of poly(dT) oligonucleotides. ET\textsubscript{B} receptor oligonucleotide probes (sense: 5’-TTCACCTCAGCAGGATTCTG-3’; antisense: 5’-AGGTTGGAAGTTAGAACG-3’) were synthesized from the published probe sequence of Wang and colleagues,\textsuperscript{26} who verified their specificity by sequencing the PCR product. The probes of \( \beta \)-actin (as housekeeping gene) (sense: 5’-GTGGGGCCGTCTTAGGACAA-3’; antisense: 5’-CTCTTTG-ATGTACGCGACGATTCT-3’) were synthesized according to the published sequence of Ali and colleagues.\textsuperscript{27} Amplification conditions with the use of a thermocycler (Eppendorf 53.32) were the following: 95°C (40 seconds), 55°C (40 seconds), and 72°C (1 minute). The number of cycles was 27 for \( \beta \)-actin and 35 for the ET\textsubscript{B} receptor. Amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide.

Measurement of Infarct Volume

Under deep anesthesia, 24 hours after the insult, rats were killed, and the brains were removed rapidly and frozen in cooled isopentane at −65°C for 15 seconds. Whole brains were cut in 20-μm-thick sections with a cryostat (Leica CM3050), and 1 section in every 40 was collected on a glass slide and stained with thionin. The infarcted area was quantified by image analysis (Scion Image, See Scan). Infarction volume, corrected for edema as described previously,\textsuperscript{28} was calculated by the integration of infarct areas over 16 equidistant brain slices that encompassed the whole lesion.

Data and Statistical Analysis

The results are presented as mean±SEM. Statistical analyses were performed with ANOVA followed by the Bonferroni-Dunn test as indicated. \( P<0.05 \) was accepted as significant.

Results

ET\textsubscript{B} mRNA Expression After MCAO

The expression of ET\textsubscript{B} mRNA receptors was examined through the use of the RT-PCR technique at 3 hours, 6 hours, 24 hours, and 3 days after 1-hour temporary ischemia (3 rats at each time point were used). We verified the presence of ischemia-induced brain damage using the neurological test of Bederson and coworkers\textsuperscript{29} in rats examined at 3 and 6 hours and using histology for rats examined at 24 hours and 3 days. For each experiment we obtained a single PCR product of the expected size (475 bp for ET\textsubscript{B} and 539 bp for \( \beta \)-actin). The expression of ET\textsubscript{B} receptor mRNA in the ipsilateral hemisphere, at the time points analyzed, showed no change after MCAO compared with that observed in the respective contralateral hemisphere and with sham-operated animals. The housekeeping gene \( \beta \)-actin also showed no change over time (Figure 1). Similarly, the lesion induced by NMDA injection

NMDA-Induced Excitotoxic Damage

In halothane-anesthetized rats, excitotoxic lesions were induced by a microinjection (2 \mu L) into the right parietal cortex (coordinates 5 mm lateral, 3.4 mm ventral to the bregma)\textsuperscript{30} of NMDA (50 nmol) at a rate of 1 \mu L/min. The lesion volume was quantified 24 hours after the injection of NMDA.
did not result in any changes in ET$_B$ mRNA expression as examined at 1, 12, and 24 hours after the injection (data not shown).

**Effects of BQ-788 on CBF and Brain Damage**

In pilot experiments, we tested the effect of 2 doses of BQ-788 (4 and 40 µg) administered into the lateral ventricle, 30 minutes before and 30 minutes after MCAO, on brain damage. The volumes of hemispheric infarction were 78.5 ± 13.8, 141.4 ± 28.3, and 156.6 ± 20.3 mm$^3$ in rats treated with vehicle (n=8), BQ-788 at a dose of 4 µg (n=9), and BQ-788 at a dose of 40 µg (n=9), respectively. The ET$_B$ antagonist elicited a statistically significant increase in infarction volume ($P=0.029$) only when a dose of 40 µg was used ($P=0.076$ for the dose of 4 µg). We subsequently used the dose 40 µg in other groups of animals in which physiological parameters as well as CBF were monitored.

Physical parameters (mean arterial pressure, PaCO$_2$, PaO$_2$, pH, and body temperature) were not different in BQ-788–treated animals (n=8) compared with those treated with vehicle (n=7) (Table). In these animals and as found in the pilot experiments, BQ-788 injected into the lateral ventricle increased dramatically the volume of infarction, essentially in the cortex, compared with vehicle (178%; $P=0.027$) (Figure 2A and 2B). BQ-788 also increased the volume of brain edema, although statistical significance was not reached ($P=0.14$) (Figure 2C). BQ-788 per se administered into the ventricle failed to induce any discernible lesion in nonischemic animals.

The analysis of LDF data showed that BQ-788 did not affect the basal CBF during the 30-minute period before MCAO ($P=0.2$) (Figure 3A). MCAO induced a similar initial decrease in CBF in both BQ-788– and vehicle-treated rats (Figure 3A). The CBF decrease remained stable during the whole period of ischemia in vehicle-treated animals. However, a further decrease in CBF in animals treated with BQ-788 was observed in the second 30-minute period of MCAO (Figure 3A). The comparison of CBF values measured during the second 30-minute period of ischemia showed a statistically significant difference between BQ-788– and vehicle-treated animals (–26.7%; $P=0.006$, ANOVA followed by Bonferroni-Dunn test) (Figure 3B). After reperfusion, hyperemia was observed, and CBF returned to preocclusion values after 15 minutes in a similar manner in both groups ($P=0.15$) with, however, high interindividual variability (Figure 3B). In addition, there was a significant correlation ($P<0.001$) between the volume of infarction and the change in residual CBF during MCAO (Figure 4).

**Effects of BQ-788 on NMDA-Induced Excitotoxic Brain Damage**

The glutamatergic agonist NMDA (50 nmol) produced a well-defined cortical lesion 24 hours after injection (Figure 5B). Administration of BQ-788 (40 µg) into the lateral ventricle 30 minutes before and 30 minutes after NMDA injection failed to change the volume of cortical damage (39.7 ± 7.5 mm$^3$ for NMDA+vehicle [n=6] and 37.3 ± 7.6 mm$^3$ for NMDA+BQ-788 [n=6]) (Figure 5A). Physiological parameters (mean arterial pressure, PaCO$_2$, PaO$_2$, pH, body temperature, and heart rate) were not different in BQ-788–treated animals compared with those treated with vehicle (Table).

**Physiological Parameters Before and During Ischemia or NMDA-Induced Excitotoxic Damage**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cerebral Ischemia</th>
<th>NMDA-Induced Damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle-Treated Group (n=7)</td>
<td>BQ-788–Treated Group (n=8)</td>
</tr>
<tr>
<td></td>
<td>Before Ischemia</td>
<td>During Ischemia</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
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<td>PaCO$_2$ (mm Hg)</td>
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<td>113±11</td>
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<tr>
<td>pH</td>
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<td>7.44±0.05</td>
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<tr>
<td>Temperature, °C</td>
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<td>37.6±0.1</td>
</tr>
<tr>
<td>Heart rate</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; ND, not determined.

Values are mean ± SEM. Statistics were performed by ANOVA; no significant differences were found.
Discussion

Three novel findings arise from the present study. First, cerebral ischemia failed to modify the expression of ET<sub>B</sub> receptor mRNA examined in both the acute and chronic stages of ischemia. Second, selective ET<sub>B</sub> receptor blockade dramatically exacerbated brain damage and reduced cortical perfusion after ischemia. Third, NMDA-induced excitotoxic damage was not affected by ET<sub>B</sub> receptor blockade.

Because of the potent vasoconstrictive actions of ET-1 and its upregulation after stroke, this peptide has been implicated in the pathogenesis of ischemic brain lesions. The definitive mechanisms of action of endothelin, however, are not yet fully established. Selective ET<sub>A</sub> or mixed ET<sub>A</sub>/ET<sub>B</sub> antagonists have been shown in some, but not all, studies to ameliorate CBF and neurological outcome and to reduce infarction volume in models of global and focal cerebral ischemia in rats and cats. One of the key issues is whether selective ET<sub>A</sub> or mixed ET<sub>A</sub>/ET<sub>B</sub> antagonists are likely to be effective in treating stroke.

In the cerebral circulation, it is well established that ET<sub>A</sub> mediates a potent and long-lasting constriction; however, the role of ET<sub>B</sub> receptor, in both physiological and pathophysiological conditions, remains not fully elucidated. Although the blockade of ET<sub>B</sub> receptors, under physiological conditions, does not change the diameter of cerebral arterioles, the majority of studies have shown that selective activation of ET<sub>B</sub> receptors mediates vasodilatation. Nonetheless, in some reports, cerebrovascular ET<sub>B</sub> receptor has been linked to the contractile properties of endothelins because those are more widely observed in the systemic circulation. In the present work we focused specifically on the roles of ET<sub>B</sub> receptors in focal cerebral ischemia in the rat.

Figure 2. A, Effect of BQ-788 on cerebral infarction 24 hours after transient MCAO (60 minutes). BQ-788 (40 μg; n=8) or vehicle (DMSO; n=7) was administered intracerebroventricularly 30 minutes before and 30 minutes after MCAO under monitoring of physiological parameters and CBF. Infarct volumes are expressed in cubic millimeters corrected for the volume of edema. *P<0.05 vs vehicle (ANOVA followed by Bonferroni-Dunn test). B, Distribution of the infarction at the level of the caudoputamen in the rats used in each group. C, Effect of BQ-788 on cerebral edema 24 hours after transient MCAO. BQ-788 increased the volume of edema compared with vehicle, although no statistical significance was reached (P=0.14, ANOVA).

Figure 3. A, Cortical perfusion assessed by LDF in vehicle-treated (n=7) and BQ-788–treated (n=8) animals before, during, and after transient MCAO. Data are expressed as percentage of the mean of 10-minute preocclusion values of CBF. BQ-788 (40 μg) or vehicle (DMSO) was administered intracerebroventricularly 30 minutes before and 30 minutes after MCAO. The bars indicate the time of MCAO and injection of BQ-788 or its vehicle. B, Residual CBF assessed by LDF during the first and the second 30-minute epochs after MCAO in BQ-788–treated and control rats. (**P<0.01, ANOVA followed by Bonferroni-Dunn test).
The analysis of the kinetics of expression of ET\textsubscript{B} receptors mRNA in the ipsilateral hemisphere showed no change through the acute and chronic stages of ischemia. In each rat, the presence of ischemia-induced lesion was verified by neurological tests\textsuperscript{29} for acute time points (3 and 6 hours) and by histological analysis for chronic time points (24 hours and 3 days). The absence of changes in expression observed through the acute and chronic stages may be due to the heterogeneity of brain tissue used for this analysis, which probably encompasses regions with different degrees of ischemia (ie, severely ischemic tissue, penumbral tissue, and healthy tissue). However, we have previously observed up-regulation of some elements of the endothelin system, such as ET-1 and ETA receptors, in the same rats using the same technique (ie, RT-PCR) as in the present study.\textsuperscript{38} In global cerebral ischemia, Yamashita and coworkers\textsuperscript{39} reported an increase in ET\textsubscript{B} receptor binding in microglia in the hippocampal pyramidal cell field 7 days after global ischemia. The authors suggested that ET-1 released from astrocytes, in response to ischemia, would activate microglia with ET\textsubscript{B} receptors to participate in neuronal death in the hippocampus. Moreover, an increase in ET\textsubscript{B} receptor agonist binding sites and ET\textsubscript{B} mRNA receptors after experimental subarachnoid hemorrhage has been shown.\textsuperscript{40–44} Our findings and the aforementioned results show that ET\textsubscript{B} receptors are present in vascular and nonvascular brain tissue after cerebrovascular insults. However, the functionality and the significance of these receptors cannot be ascertained from these descriptive data.

The present study provides the first definitive evidence that blockade of ET\textsubscript{B} receptor enhances ischemic brain damage. Our data demonstrate that intracerebroventricular administration of the well-characterized ET\textsubscript{B} antagonist BQ-788 remarkably increased the volume of infarction (178\%) as well as the volume of edema (200\%) and significantly decreased the residual cortical CBF (−26.7\%). BQ-788 per se is not toxic in the normal brain.\textsuperscript{20}

Two potential mechanisms may explain the ET\textsubscript{B} blockade–induced exacerbation of ischemic brain lesion. First, given the direct vasomotor actions mediated by ET\textsubscript{B} receptor, one would assume that BQ-788 counteracts the relaxing actions of ET\textsubscript{B} during ischemia. In this case, the overall effects of postischemic endogenous ET-1 would be the result of a balance between ETA-mediated vasoconstriction and ET\textsubscript{B}-mediated vasodilatation. Moreover, it has been reported in many peripheral tissues, eg, heart and lung, that ET\textsubscript{B} receptor can act as a clearance receptor for ET-1.\textsuperscript{42–44} If this occurs in the brain, where ET\textsubscript{B} receptors are widely expressed, mainly in astrocytes, treatment with BQ-788 would increase the availability of the postischemic released ET-1 for ETA receptors. This would also result in enhancement of vasoconstriction.

Our data support these ET\textsubscript{B}-mediated vascular effects since the blockade of this receptor resulted in an exacerbation of MCAO-induced hypoperfusion. In addition, there was a good correlation between the volume of infarction and changes in

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**Figure 4.** Correlation between infarction volume and changes in residual CBF. For each animal, CBF is expressed as the percent changes in residual CBF between the second 30-minute period (ie, 30 to 60 minutes of MCAO) and the first 30-minute period (ie, 0 to 30 minutes of MCAO) of ischemia. ($R^2=0.62$; $P<0.001$, ANOVA regression analysis).

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**Figure 5.** A, Failure of BQ-788 to modify the volume of the lesion produced by cortical NMDA (50 nmol) injection ($n=6$ in every group). BQ-788 (40 μg) or vehicle (DMSO) was administered intracerebroventricularly 30 minutes before and 30 minutes after NMDA injection under monitoring of physiological parameters. Lesion volumes are expressed in cubic millimeters. B, Topography of the lesion induced by NMDA at the level of the caudoputamen in the rats used in each group.
residual CBF. The available data indicate that the fate of the penumbral tissue, characterized by a relatively mild hypoperfusion, could be determined by subtle changes in CBF.\(^{45}\) Moreover, the failure of BQ-788 to modify the lesion size in NMDA-induced brain injury, a model in which excitotoxicity is the main factor that induces cell death and in which vascular mechanisms are not predominant,\(^ {46} \) reinforces the idea that ET\(_B\) antagonism likely enhances ischemic damage through its action on the cerebral circulation.

Second, BQ-788 could act on nonvascular tissue to enhance deleterious events in ischemic tissue. The mammalian brain predominantly contains the ET\(_B\) receptor subtype expressed mainly on endothelial and glial cells.\(^ {47-49} \) This widespread nonvascular distribution of ET\(_B\) receptors suggests a role for ET receptors in nonvascular functions. It has been suggested that endothelins act, through ET\(_A\), as growth factors for astrocytes, regulating biological processes such as proliferation during brain development or injury.\(^ {6,50-52} \) Ho and coworkers\(^ {53} \) demonstrated that astrocytes without ET-1 are more vulnerable to hypoxic/ischemic injuries and that upregulation of astrocytic ET-1 is essential for the survival of astrocytes. The roles of ET\(_A\) in nonvascular brain tissue after experimental ischemia would be best addressed in brain cellular cultures devoid of vasculature.

Whatever the mechanisms underlying BQ-788-mediated effects in cerebral ischemia, our data suggest that the optimal therapeutic strategy in stroke targeting endothelins would be the use of selective ET\(_B\) antagonists and not mixed ET\(_A/ET_B\) antagonists.

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References


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