Neuroprotective Effects of (S)-N-[4-[4-[3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl]carbonyl]-1-piperazinyl]phenyl]-2-thiophenecarboximid-amide (BN 80933), an Inhibitor of Neuronal Nitric-Oxide Synthase and an Antioxidant, in Model of Transient Focal Cerebral Ischemia in Mice

LI DING-ZHOU, CATHERINE MARCHAND-VERRECCHIA, BRUNO PALMIER, NICOLE CROCI, PIERRE-ETIENNE CHABRIER, MICHEL PLOTKINE, and ISABELLE MARGAILL

Laboratoire de Pharmacologie, Université René Descartes, Paris, France (L.D.Z., C.M.V., B.P., N.C., M.P., I.M.); and Beaufour-Ipsen Research Laboratories, Institut Henri Beaufour, Les Ulis, France

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ABSTRACT
Nitric oxide (NO) and reactive oxygen species are both implicated in neuronal death due to cerebral ischemia. BN 80933, an original compound associating an inhibitor of neuronal NO synthase with an antioxidant, has been shown to reduce functional and histological damage in rat submitted to cerebral ischemia. The aim of the present study was to confirm these results in mice and to further examine the effects of BN 80933 on inflammatory response, including blood-brain barrier (BBB) disruption, brain edema, and neutrophil infiltration after transient middle cerebral artery occlusion (MCAO). Intravenous administration of BN 80933 at 3 and 10 mg/kg 3 h after MCAO significantly reduced by 26 to 36% the infarct volume evaluated 24 and 48 h after ischemia, and improved the neurological score. Furthermore, BN 80933 at both dosages decreased by 42 to 75% the extravasation of Evans blue in brain parenchyma observed 24 h after ischemia. This reduction in BBB disruption was associated with decreased brain edema as demonstrated by the 37% reduction in brain water content induced by BN 80933 at 3 mg/kg 24 h after MCAO. Neutrophil infiltration in brain parenchyma, evaluated by the myeloperoxidase activity, was also reduced by 45 to 56% in animals treated with BN 80933 at 3 and 10 mg/kg. Together, these results extend the protective capacity of BN 80933 against brain ischemic injury and confirm that BN 80933 represents a promising treatment for stroke.

Transient focal cerebral ischemia enhanced the formation of free radicals, including reactive oxygen species (ROS) and nitric oxide (NO) in brain tissue. Both ROS and NO are known to trigger molecular pathways that lead to neuronal loss and serious motor disturbances (Dirnagl et al., 1999). These species can act independently as well as cooperatively by forming the highly reactive oxidant peroxynitrite (Beckman et al., 1990). Besides classical pharmacological studies, molecular genetic approaches using mutant mice overexpressing/deficient in the major endogenous enzymes of the free radical pathway (e.g., superoxide dismutase and glutathione peroxidase) and mice lacking neuronal nitric-oxide synthase (nNOS) have given evidence of the implication of ROS and nNOS-derived NO in ischemic damage (Lewen et al., 2000; Chan, 2001). Therefore, NO synthase inhibitors (Iadecola, 1997; Chabrier et al., 1999b) and scavengers of ROS (Cuzzocrea et al., 2001) have been proposed as strategies for neuroprotection in stroke. A combined treatment associating an NO inhibitor and an antioxidant/superoxide anion scavenger provides a synergistic neuroprotective effect in transient focal ischemia in rats (Spinnewyn et al., 1999). On the basis of this observation, Chabrier et al. (1999a) have developed a new therapeutic concept combining, in the same

ABBREVIATIONS: ROS, reactive oxygen species; NO, nitric oxide; nNOS, neuronal nitric-oxide synthase; NOS, nitric-oxide synthase; BBB, blood-brain barrier; MCAO, middle cerebral artery occlusion; BWC, brain water content; MPO, myeloperoxidase; BN 80933, (S)-N-[4-[4-[3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl]carbonyl]-1-piperazinyl]phenyl]-2-thiophenecarboximid-amide; MABP, mean arterial blood pressure; ANOVA, analysis of variance; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; PaO2, partial pressure in arterial oxygen; PaCO2, partial pressure in arterial carbon dioxide.
molecule, activities inhibiting nNOS and lipid peroxidation. BN 80933, a representative of this novel class of agent, was shown to markedly reduce infarct volume and to improve neurological signs in transient focal ischemia in rat (Chabrier et al., 1999a). In this context, we first investigated the effects of BN 80933 on functional and histological outcomes in mice submitted to transient focal ischemia. Then we focused on the effects of BN 80933 on other postischemic damage implicating oxidative stress: opening of blood-brain barrier (BBB), brain edema formation, and neutrophil infiltration.

Materials and Methods

Chemicals

BN 80933 ((S)-N-[4-[4-[3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl]carbonyl]-1-piperazinyl]phenyl]-2-thiophene carbimidamide) (Chabrier et al., 1999a) was synthesized in the Medicinal Chemistry Department of Institut Henri Beaufour (Les Ulis, France). BN 80933 was dissolved in 5% glucose and injected in the tail vein (10 ml/kg) in all experiments. Paraformaldehyde was purchased from Sigma Chemical (Saint Quentin Fallavier, France); this value was multiplied by the ratio of the surface of the infarct hemisphere to the intact hemisphere to correct the lesion for brain swelling. Infarct volume, expressed in cubic millimeters, was calculated by a linear integration of the corrected lesion areas.

Blood-Brain Barrier Permeability

The integrity of BBB was investigated using Evans blue extravasation as described previously (Ding-Zhou et al., 2002). Evans blue at 1% in saline (100 µl) was injected intravenously and allowed to circulate for 60 min. Animals were anesthetized with pentobarbitone (55 mg/kg i.p.) and perfused transcardially with saline at 100 mm Hg pressure until blue color was absent from the effluent. Mice were decapitated and brains were immediately removed and placed on a frozen plate. Tissue samples were dissected out from infarct areas in ischemic mice and from corresponding areas in sham-operated and nonoperated animals. Samples were weighed and placed in 400 µl of pure formamide and incubated for 72 h in the dark at 50°C. The optical density of the formamide solution was measured at 620 nm. Data are expressed as micrograms of Evans blue per gram of tissue.

Cerebral Edema

Cerebral edema was determined by measuring the brain water content (BWC) according to the wet-dry method (Hatashita et al., 1988). Brains were removed, and tissue samples were dissected out as described above. Samples were immediately weighed to obtain wet weight. Samples were then dried in a desiccator at 110°C for 24 h and weighed again to obtain the dry weight. BWC, expressed as percentage, was calculated as follows: BWC (%) = (wet weight − dry weight) × 100/wet weight.

Neutrophil Infiltration

Myeloperoxidase (MPO) activity was used as an indicator of neutrophil infiltration in brain parenchyma (Barone et al., 1991). Mice were anesthetized with sodium pentobarbitone (55 mg/kg i.p.) and transcardially perfused with 200 ml of saline at 20°C with a pressure of 100 mm Hg to flush blood components from the vasculature. Brains were removed and samples dissected out as previously described were immediately frozen at −40°C. MPO activity was determined as described by Barone et al. (1991) and modified by Batteur-Parmentier et al. (2000). One unit of MPO activity is defined as the amount that degrades 1 µmol of hydrogen peroxide per minute at 25°C, and normalized to the weight of wet tissue (units of MPO per gram of tissue).

Experimental Design

Effect of BN 80933 on Neurological Deficit and Infarct Volume

BN 80933 at 3 and 10 mg/kg or its vehicle was injected 3 h after MCAO. The effect of BN 80933 on neurological deficit and infarct volume was evaluated 24 h after ischemia in the first experiment and

Neurological Deficit

Sensorimotor neurological deficits were assessed by a grip test (Hall, 1985). Mice were picked up by the tail and placed on a taut string, 60 cm in length, suspended 40 cm above a table. Grip score was measured as the length of time that the mouse remained on the string in some manner (using one or more paws, tail, tail plus paws), for a maximum of 30 s. Each experiment was conducted randomly and blindly.

Infarct Volume

After neurological deficit evaluation, mice were killed with an overdose of sodium pentobarbitone (200 mg/kg i.p.) for infarct volume determination as described previously (Ding-Zhou et al., 2002). The brain was removed and sectioned coronally into six 1-mm-thick slices using a tissue Chopper (Mcllwain, The Mickle Laboratory Engineering Co., Gomshall, Surrey, UK). Coronal brain slices were immediately immersed in 2% 2,3,5-triphenyltetrazolium chloride for 20 min at room temperature in the dark followed by fixation in a 4% paraformaldehyde solution overnight before analysis. The infarction area, outlined in white, was measured on the posterior surface of each section using a computer image analysis system (Imstar, Paris, France); this value was multiplied by the ratio of the surface of the infarct hemisphere to the intact hemisphere to correct the lesion for brain swelling. Infarct volume, expressed in cubic millimeters, was calculated by a linear integration of the corrected lesion areas.
48 h after ischemia in the second experiment. In both studies, the neurological score of nonoperated and sham-operated mice was also determined.

Effect of BN 80933 on BBB Permeability. Mice were assigned to five groups: a nonoperated group, a sham-operated group, and three ischemic groups treated 3 h after MCAO with BN 80933 at 3 or 10 mg/kg or with its vehicle. The effect of BN 80933 on BBB permeability was evaluated 24 h after the surgical process on the basis of our previous study showing Evans blue extravasation at this time point (Ding-Zhou et al., 2002).

Effect of BN 80933 on Cerebral Edema. BN 80933 at 3 and 10 mg/kg or its vehicle was injected 3 h after MCAO, and BWC was measured 24 h after ischemia. BWC was also measured in nonoperated and sham-operated mice.

Temporal Evolution of Brain MPO Activity after Transient MCAO. MPO activity was measured in ischemic and in sham-operated mice 1, 2, 3, 5, and 7 days after the onset of the surgical process. Nonoperated mice were concomitantly studied.

Effect of BN 80933 on MPO activity. BN 80933 at 3 and 10 mg/kg or its vehicle was injected 3 h after MCAO; MPO activity was measured 48 h after MCAO in ischemic mice and corresponding sham-operated mice and nonoperated mice.

Effect of BN 80933 on Physiological Variables. A catheter was inserted into the common carotid artery and connected to a pressure transducer (Emka Technologies, Paris, France) to monitor the mean arterial blood pressure (MABP). Once MABP was stabilized (basal level), animals were treated with BN 80933 at 3 and 10 mg/kg or with its vehicle, and the MABP was recorded for 2 h. Blood samples were collected before and 30, 60, and 120 min after treatment. Analysis of arterial blood gases and pH, partial pressure in arterial oxygen (PaO2), and arterial carbon dioxide (PaCO2), using a blood gas/pH analyzer (ABL 330; Radiometer, Nevilly-Plaisance, France). Body temperature was maintained at 37 ± 0.5°C by the means of a heating blanket.

Data Analysis. Data are expressed as means ± S.E.M. of n observations, where n represents the number of animals or samples. Comparison between multiple groups was evaluated by a one-way analysis of variance (ANOVA) followed by a PLSD (protected least significant difference) Fisher’s test. For the time course study, the effect of the surgical procedure was first evaluated by comparing the sham-operated groups to the nonoperated group using a one-way ANOVA followed by Dunnett’s test. Comparisons between sham-operated and ischemic groups were then performed by a one-way ANOVA followed by a protected least significant difference Fisher’s test. When measures were repeated (physiological variables), data were analyzed by a two-factorial ANOVA for repeated measure. A p value of less than 0.05 was considered to be statistically significant.

Results

Effect of BN 80933 on Neurological Deficit and Infarct Volume

Experiment 1. Twenty-four hours after ischemia, the grip score was maximal in sham-operated mice (30.0 ± 0.0 s) and was not significantly different from that of nonoperated mice (29.8 ± 0.3 s). Transient MCAO led to a dramatic decrease in the grip score (10.5 ± 3.4 s, p < 0.001 compared with sham-operated mice). BN 80933 given 3 h after MCAO significantly improved the grip score at 3 mg/kg (26.8 ± 3.1 s, p < 0.001) and 10 mg/kg (23.1 ± 3.1 s, p < 0.01) compared with vehicle-treated mice (Fig. 1a). Total infarct volume was 90 ± 5 mm³ in vehicle-treated ischemic mice. BN 80933 reduced the lesion by 31% at 3 mg/kg (62 ± 8 mm³, p < 0.01) and by 36% at 10 mg/kg (58 ± 5 mm³, p < 0.01) (Fig. 1b). Representative coronal brain sections of vehicle- and BN 80933-treated ischemic mice are given in Fig. 2 and show that BN 80933 reduced the infarct area by 75% at 3 mg/kg (1.7 ± 0.4 µg/g tissue, p < 0.001) and by 55% at 10 mg/kg (0.9 ± 0.4 µg/g tissue, p < 0.01) compared with vehicle-treated ischemic mice (74 ± 5 mm³) (Fig. 3, a and b).

Effect of BN 80933 on Blood-Brain Barrier Permeability

Evans blue content in the brain of nonoperated mice was very low (0.5 ± 0.2 µg/g tissue) and was not significantly different from sham-operated mice (0.7 ± 0.4 µg/g tissue) (Fig. 4). Twenty-four hours after MCAO, Evans blue content in the ischemic area of vehicle-treated group was markedly increased compared with sham-operated mice (6.9 ± 0.8 µg/g tissue, p < 0.001). BN 80933 reduced the Evans blue level of infarct area by 75% at 3 mg/kg (1.7 ± 0.7 µg/g tissue, p < 0.001) and by 42% at 10 mg/kg (4.0 ± 1.4 µg/g tissue, p < 0.05) compared with vehicle-treated ischemic mice (74 ± 5 mm³) (Fig. 3, a and b).

Effect of BN 80933 on Brain Edema Formation

The BWC in sham-operated mice was 79.7 ± 0.7%, which was not significantly different from nonoperated mice (80.0 ± 0.7%) (Fig. 5). Twenty-four hours after MCAO, the BWC in the ischemic area of the vehicle-treated group

Fig. 1. Effect of BN 80933 on neurological deficit (a) and infarct volume (b) 24 h after transient MCAO. BN 80933 at doses of 3 and 10 mg/kg was given (i.v.) 3 h after MCAO. Data are means ± S.E.M. (n = 8–9/group). ***p < 0.001 versus sham-operated mice. †††, p < 0.001 and ††, p < 0.01 versus vehicle-treated ischemic mice.

80933 at 3 and 10 mg/kg reduced both cortical and subcortical lesions.

Experiment 2. Forty-eight hours after ischemia, BN 80933 given 3 h after MCAO at doses of 3 and 10 mg/kg improved the grip score (respectively, 21.9 ± 2.8 s, p < 0.05 and 22.4 ± 3.2 s, p < 0.05 versus 13.9 ± 3.6 s in vehicle-treated ischemic mice) and reduced the infarct volume by 31% at 3 mg/kg (51 ± 6 mm³, p < 0.01) and by 26% at 10 mg/kg (55 ± 6 mm³, p < 0.05) compared with vehicle-treated ischemic mice (74 ± 5 mm³) (Fig. 3, a and b).

Effect of BN 80933 on Cerebral Edema Formation

The BWC in sham-operated mice was 79.7 ± 0.7%, which was not significantly different from nonoperated mice (80.0 ± 0.7%) (Fig. 5). Twenty-four hours after MCAO, the BWC in the ischemic area of the vehicle-treated group
(86.2 ± 0.6%) was significantly higher than that of the sham-operated mice (p < 0.001). BN 80933 significantly reduced brain edema at 3 mg/kg (83.8 ± 0.9%, p < 0.05 versus vehicle-treated mice). At 10 mg/kg, BN 80933 also tended to decrease the brain water content but the difference did not reach significant level (84.0 ± 1.0%, p = 0.07 versus vehicle-treated mice).

Temporal Evolution of Brain MPO Activity after Transient MCAO

MPO activity in the brain of nonoperated mice was 0.016 ± 0.006 U/g tissue and was not significantly different from that of sham-operated mice (Fig. 6). The level of MPO activity in the infarct area reached 0.234 ± 0.042 U/g tissue 2 days after ischemia (p < 0.001 versus corresponding sham-operated mice) and remained elevated at 3 days (0.238 ± 0.064 U/g tissue, p < 0.001). MPO activity was returned to baseline 5 days after MCAO.

Effect of BN 80933 on MPO Activity

MPO activity in sham-operated mice (0.015 ± 0.005 U/g tissue) was not significantly different from nonoperated mice (0.015 ± 0.004 U/g tissue) (Fig. 7). Two days after ischemia, vehicle-treated mice exhibited a massive increase in MPO activity (0.144 ± 0.031 U/g tissue, p < 0.001 versus sham-operated mice). BN 80933 reduced MPO activity by 56% at 3 mg/kg (0.064 ± 0.013 U/g tissue, p < 0.01) and by 45% at 10 mg/kg (0.078 ± 0.022 U/g tissue, p < 0.05) compared with the vehicle-treated group.

Effect of BN 80933 on Physiological Variables

The physiological variables measured before and after administration of BN 80933 (3 and 10 mg/kg) and its vehicle are summarized in Table 1. Arterial blood gases, pH, and MABP remained within the physiological ranges throughout the experiment and were not significantly different between the experimental groups.
Discussion

The present study demonstrated that BN 80933, an inhibitor of nNOS and lipid peroxidation, reduces infarction and improves the neurological score of mice submitted to transient focal cerebral ischemia. Furthermore, our results show for the first time that BN 80933 reduces the damage to the BBB and decreases cerebral edema formation and neutrophil infiltration.

The role of NO generated by the three NO synthase isoforms (nNOS, endothelial NOS or eNOS, and inducible NOS or iNOS) in focal cerebral ischemia is now well documented (Iadecola, 1997). The use of selective NOS inhibitors and knockout mice deficient in one NOS isoform indeed demonstrated that excessive NO production by nNOS (Huang et al., 1994; Hara et al., 1996) and iNOS (Iadecola et al., 1997) contributes to cell damage, whereas NO generated from eNOS is neuroprotective (Huang et al., 1996).

Evidence has accumulated showing that the massive generation of reactive free radicals during reperfusion and the resulting formation of peroxynitrite cause lipid peroxidation, protein oxidation, and DNA damage (Kuroda and Siesjo, 1997; Chan, 2001). Recently, both animal and human studies have provided evidence that the oxidative damage to membrane lipids and proteins is increased during ischemia and reperfusion (Mason et al., 2000). Pharmacological approaches using scavengers of free radicals or mimics of endogenous antioxidant enzymes such as superoxide dismutase and transgenic or knockout mutant mice demonstrated the injurious role of ROS in cerebral ischemia (Lewen et al., 2000; Cuzzocrea et al., 2001).

In view of the deleterious effect of nNOS-generated NO
and ROS production in cerebral ischemia, Chabrier et al. (1999a) had the idea to combine in one compound antioxidant and nNOS inhibitory properties. Accordingly, BN 80933 associated a benzopyran moiety (Troxol derivative) with thioephene amidine via a piperazine link. The antioxidant effect of BN 80933 was first demonstrated in vitro (Chabrier et al., 1999a) but was also confirmed in vivo (Marin et al., 2000). BN 80933 indeed reduced the production of 8-epi-prostaglandin F2α, a marker of oxidative stress after transient focal cerebral ischemia in rats. With regard to its nNOS inhibitory effect, BN 80933 was demonstrated in vitro as a competitive inhibitor (Kᵢ = 0.92 μM) with a high selectivity (120-fold versus eNOS, more than 300-fold versus iNOS) (Chabrier et al., 1999a).

In the first step of our study, we examined from a functional and histological point of view the effect of BN 80933 in mice submitted to transient MCAO. The duration of MCA occlusion used in our experiments was chosen according to a previous report by Huang et al. (1999) showing that a 15-min occlusion with a 6-0 suture induced severe ischemia followed by partial reperfusion. In a preliminary experiment, we observed that blood flow in the MCA territory was reduced to 15% of the pres ischemic value during ischemia and that it returned to 50% of the pres ischemic value 1 h after the onset of reperfusion (L. Ding-Zhou and I. Margaill, unpublished data). This progressive and incomplete reperfusion may be partly due to vascular damage induced by thread insertion and retraction, as reported previously (Huang et al., 1999). It may explain why, despite the short ischemia duration, large infarct volumes, involving both cortical and subcortical structures, were observed in our experiments.

The dosing regimen we used for BN 80933 was based on previous studies in which transient MCAO was performed in rats (Chabrier et al., 1999a; Marin et al., 2000). We found that BN 80933 markedly reduced the neurological deficit in mice, as it did in rats. Furthermore, this improvement of neurological function was associated with a decrease in the total infarct volume; this decrease concerned both the cortical and subcortical regions. These beneficial effects were observed 24 h after ischemia and were still present at 48 h. It is noteworthy that even at the higher dose used BN 80933 did not increase MABP, demonstrating that this agent did not alter eNOS. These results corroborate the neuroprotective potential of BN 80933 demonstrated in transient focal cerebral ischemia in rats, global ischemia in gerbils, and traumatic brain injury in mice (Chabrier et al., 1999a).

In the second step, we focused on the effects of BN 80933 on delayed inflammatory phenomena triggered by ROS and NO. It is well established that BBB is one of the targets for ROS and may explain why, despite the short ischemia duration, large infarct volumes, involving both cortical and subcortical structures, were observed in our experiments.
be neuroprotective not only by directly acting on neurons but also by reducing the consequences of vascular damage.

It would be interesting in future experiments to determine the therapeutic window of BN 80933 and to evaluate its benefit compared with an nNOS inhibitor and an antioxidant. Using the same model, we already reported that N-nitro-l-arginine methyl ester has a similar effect on infarction, neurological deficit, and BBB disruption (Ding-Zhou et al., 2002). In contrast, N-nitro-l-arginine methyl ester was shown to have no effect on neutrophil infiltration in transient focal cerebral ischemia in rats, or even to increase it (Batteur-Parmentier et al., 2000).

In conclusion, BN 80933, a dual inhibitor of NO production and lipid peroxidation, could be a promising drug for stroke therapy because it may act at various levels.

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References

Address correspondence to: Dr. Isabelle Marguill, Laboratoire de Pharmacologie, Université René Descartes, 4 Avenue de l’Observatoire, 75006 Paris, France. E-mail: marguill@pharmac.munic-paris.fr.