A Newly Synthesized Poly(ADP-Ribose) Polymerase Inhibitor, DR2313 [2-Methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one]: Pharmacological Profiles, Neuroprotective Effects, and Therapeutic Time Window in Cerebral Ischemia in Rats

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ABSTRACT
We investigated the pharmacological profiles of DR2313 [2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one], a newly synthesized poly(ADP-ribose) polymerase (PARP) inhibitor, and its neuroprotective effects on ischemic injuries in vitro and in vivo. DR2313 competitively inhibited poly(ADP-ribosylation) in nuclear extracts of rat brain in vitro ($K_i = 0.23 \mu M$). Among several NAD$^+$-utilizing enzymes, DR2313 was specific for PARP but not selective between PARP-1 and PARP-2. DR2313 also showed excellent profiles in water solubility and rat brain penetrability. In in vitro models of cerebral ischemia, exposure to hydrogen peroxide or glutamate induced cell death with overactivation of PARP, and treatment with DR2313 reduced excessive formation of poly(ADP-ribose) and cell death. In both permanent and transient focal ischemia models in rats, pretreatment with DR2313 (10 mg/kg i.v. bolus and 10 mg/kg/h i.v. infusion for 6 h) significantly reduced the cortical infarct volume. To determine the therapeutic time window of neuroprotection by DR2313, the effect of post-treatment was examined in transient focal ischemia model and compared with that of a free radical scavenger, MCI-186 (3-methyl-1-phenyl-2-pyrazolone-5-one). Pretreatment with MCI-186 (3 mg/kg i.v. bolus and 3 mg/kg/h i.v. infusion for 6 h) significantly reduced the infarct volume, whereas the post-treatment failed to show any effects. In contrast, post-treatment with DR2313 (same regimen) delaying for 2 h after ischemia still prevented the progression of infarction. These results indicate that DR2313 exerts neuroprotective effects via its potent PARP inhibition, even when the treatment is initiated after ischemia. Thus, a PARP inhibitor like DR2313 may be more useful in treating acute stroke than a free radical scavenger.

The excessive release of the excitotoxic amino acid glutamate plays a critical role in the pathogenesis of neuronal cell death following cerebral ischemia. NO produced by nNOS has been implicated in N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity of glutamate in cerebral ischemia (Dawson and Dawson, 1998). NMDA receptor activation causes an increase in intracellular calcium concentration leading to activation of nNOS. A part of the neurotoxicity evoked by NO is a result of the reaction of NO with superoxide anion to form a highly toxic radical, peroxynitrite (Dawson and Dawson, 1998; Pieper et al., 1999). Postischemic injury is also exacerbated by the increased formation of hydroxyl radical, the most reactive molecule of all oxygen radicals (Chan, 2001). These reactive oxygen species (ROS) can contribute to the DNA strand breakage, which is followed by overactivation of poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) (Szabo and Dawson, 1998; Pieper et al., 1999). PARP-1 is an abundant nuclear protein which is activated

ABBREVIATIONS: NO, nitric oxide; nNOS, neuronal NO synthase; NMDA, N-methyl-D-aspartate; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); 3AB, 3-aminobenzamide; PND, 6(5H)-phenantridione; DIQ, 1.5-dihydroxyisoquinoline; DPQ, 3,4-dihydro-5-[4-(piperidinyl)butoxy]-1(2H)-isoquinolinone; DR2313, 2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one; MCI-186, 3-methyl-1-phenyl-2-pyrazolone-5-one; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; TCA, trichloroacetic acid; EF-2, elongation factor 2; TBARS, thiobarbituric acid reactive substance; PBS, phosphate-buffered saline; MCA, middle cerebral artery; pMCAo, permanent MCA occlusion; tMCAo, transient MCA occlusion; RP-HPLC, reverse-phase high-performance liquid chromatography; MABP, mean arterial blood pressure; HR, heart rate; ANOVA, analysis of variance.
by DNA strand breakage and catalyzes the covalent attachment of poly(ADP-ribose) (PAR) from NAD^+ (s) to numerous nuclear proteins including histones and PARP-1 itself (de Murcia and Menissier de Murcia, 1994; D’Amours et al., 1999). Besides PARP-1, the other six PARPs have been identified as sPARP, PARP-2, PARP-3, tankylase-1 and -2, and VPARP (Chiarugi, 2002). With moderate levels of DNA damage, PARP-1 is thought to participate in the DNA repair process (D’Amours et al., 1999). On the other hand, overactivation of PARP-1 induced by extensive DNA damage consumes NAD^+, and consequently, ATP, leading to necrotic cell death (Ha and Snyder, 1999). In fact, PARP-1 null mice are extremely resistant to the ROS-mediated necrotic cell death (Eliasson et al., 1997; Endres et al., 1997). Recently, it has also been reported that PARP-1 plays a critical role in a caspase-independent apoptosis pathway mediated by apoptosis-inducing factor (Yu et al., 2002). These mechanisms of cell death underlying both necrosis and apoptosis by activation of PARP-1 have been implicated in the pathogenesis of ischemic brain injury and neurodegenerative diseases, and PARP inhibitors have been shown to be effective in animal models of stroke, traumatic injuries, and Parkinson’s disease (Virag and Szabo, 2002).

Previous reports show that numerous, chemically distinct PARP inhibitors ameliorated ischemia-reperfusion injury following focal cerebral ischemia (Virag and Szabo, 2002). The most widely used PARP inhibitor, 3-aminobenzamide (3AB), has been reported to prevent the neuronal cell death in in vitro and in vivo models of stroke (Endres et al., 1997; Tokime et al., 1998). However, 3AB is a relatively weak compound and does not readily cross blood brain barrier after peripheral administration (Andersson et al., 1994; Tokime et al., 1998). 3AB has also been reported to possess a radical scavenging property (Czapski et al., 2004). Recently, new series of PARP inhibitors, phanerandione and isooquinolinoline derivatives, have been developed. 6(5H)-phanerandione (PND), 1,5-dihydroxyisooquinoline (DIQ), and 3,4-dihydro-5-[4-(piperidinyl)butoxy]-1,2H)-isooquinolinone (DPQ) are more potent and specific inhibitors of PARP than 3AB (Moroni et al., 2001; Chiarugi et al., 2003). However, the solubility to aqueous phase is very poor, and they have to be dissolved in organic vehicle (e.g., dimethyl sulfoxide). For the treatment of acute ischemic stroke, a compound should ideally be soluble in aqueous vehicle such as saline and deliverable by bolus injection or infusion. Thus, to develop a new class of PARP inhibitor as a neuroprotectant for stroke, many efforts have been focused on enhancing the potency, improving the pharmacokinetic characteristics, and increasing the water solubility (Abdelkarim et al., 2001; Chiarugi et al., 2003; Ferraris et al., 2003; Komjati et al., 2004; Zhang et al., 2000).

In search for a new class of PARP inhibitors, we have recently discovered DR2313 [2-methyl-3,5,7,8-tetrahydrothiopyran[4,3-d]pyrimidine-4-one]. This compound is a potent, brain-penetrating, and water-soluble PARP inhibitor. In the present study, the pharmacological profiles and the neuroprotective effects of DR2313 were evaluated using in vitro and in vivo rat models of cerebral ischemia. In addition, the effect of post-treatment with DR2313 after ischemia, namely the therapeutic time window of the neuroprotection, was evaluated in the in vivo model of cerebral ischemia and compared with that of MCI-186 (3-methyl-1-phenyl-2-pyrazolone-5-one), a hydroxyl radical scavenger. MCI-186 (edaravone) is the first neuroprotectant used clinically in Japan for the treatment of stroke patients within 24 h after the onset of ischemia (Tanaka, 2002). Thus, the comparative study of DR2313 with MCI-186 may be meaningful for predicting the clinical efficacy of PARP inhibitor in acute ischemic stroke.

Materials and Methods

Materials. DR2313 was synthesized at Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd. (Yokohama, Japan). 3AB, PND, DIQ, and DPQ were purchased from Sigma-Aldrich (St. Louis, MO). MCI-186 was from TCI (Tokyo, Japan). [32P]NAD^+ was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH), baker yeast alcohol dehydrogenase (ADH), and swine muscle lactate dehydrogenase (LDH) were purchased from Roche Diagnostics (Basel, Switzerland). Dulbecco’s modified Eagle’s medium (DMEM), neurobasal medium, and B27 supplement were from Invitrogen (Carlsbad, CA). Rabbit reticulocyte lysate was from Promega (Madison, WI). Anti-poly(ADP-ribose) monoclonal antibody (10H), human recombinant PARP-1, and mouse recombinant PARP-2 were from Alexis Corporation (Läufelfingen, Switzerland). Alexa488-conjugated anti-mouse IgG was from Molecular Probes (Eugene, OR). Bacterial diptheria toxin was from Calbiochem (San Diego, CA). All other materials we used were of analytical grade.

PARP Inhibition Assay. The catalytic activity of PARP in nuclear extracts of the rat brain was measured as described previously (Tanuma and Kanai, 1982) with some modifications. Rat whole brains were homogenized in a buffer A containing 50 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 50 mM NaCl, 5 mM MgCl_2, 0.1 mM EDTA-3Na, 1 mM diethiothreitol, and 1 mM phenethylsulfonyl fluoride with a glass-Teflon homogenizer, and the fractions of nuclei were obtained by centrifugation (800 g for 10 min at 4°C). The nuclei were washed twice with buffer A, resuspended in buffer A containing 0.4 M NaCl, and then kept for 1 h on ice. The nuclear extracts were obtained by centrifugation (10,000 g for 30 min at 4°C) and stored at −80°C until use. The protein concentrations of each sample were measured by Bradford assay with bovine serum albumin as a standard. Ten micrograms of the nuclear extracts was added to the standard reaction mixture containing 100 mM Tris-HCl (pH 8.0), 10 mM MgCl_2, 250 μM NAD^+, 3.7 to 7.4 kBq [32P]NAD^+, 1 mM dithiothreitol, and 10 μg of sonicated DNA from salmon sperm with or without compounds in the total volume of 100 μl to start reaction. After 30 min of incubation at 37°C, the reactions were terminated by the addition of ice-cold 20% (w/v) trichloroacetic acid (TCA), and the acid-insoluble materials were transferred to a glass fiber filter (type GF/B; Millipore Corporation, Billerica, MA). The amount of radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). For an assessment of the enzyme kinetic mode by Dixon’s plots, NAD^+ at the three concentrations (125, 250, and 500 μM) was incubated in the reactions with or without DR2313.

Selectivity of DR2313. To determine the selectivity of DR2313 for PARP, we examined if DR2313 would inhibit the purified NAD^+ utilizing enzymes; human recombinant PARP-1, mouse recombinant PARP-2, rabbit muscle GAPDH, baker yeast ADH, and swine muscle LDH. The PARP-1 and PARP-2 inhibition assays were carried out in the same manner as described in the above section (PARP inhibition assay).

GAPDH activity was measured as described previously (Ishii et al., 1999). The mixture (200 μl) containing 100 mM triethyramine-HCl buffer (pH 7.6), 1 mM EDTA, 10 mM K2HPO4, 100 mM KCl, and 5 μg/ml GAPDH was preincubated with 0, 1, 10, or 100 μM DR2313 for 10 min at 37°C. The GAPDH reaction was started by the addition of 0.2 mM NAD^+ and 0.8 mM glyceraldehyde-3-phosphate at 25°C. The initial velocity of an increase in absorbance at 340 nm due to the formation of NADH was measured with a Hitachi U-2000 A spectrophotometer for 1 min.
ADH activity was measured as described previously (Zhang et al., 2000) with some modifications. The mixture (200 µl) containing 50 mM Bicine buffer (pH 7.6), 2 mM NAD+, and 1.25 µg/ml ADH was preincubated with 0, 1, 10, or 100 µM DR2313 for 10 min at 37°C. The ADH reaction was started by the addition of 5 mM ethanol at 37°C. The initial velocity was measured in the same way as in the GAPDH assay.

LDH activity was also measured as described previously (Zhang et al., 2000), with some modifications. The mixture (300 µl) containing 83 mM potassium phosphate buffer (pH 7.4), 2 mM NADH, and 3.3 µM/mg LDLH was preincubated with 0, 1, 10, or 100 µM DR2313 for 10 min at 37°C. The LDH reaction was started by the addition of 16.8 mM pyruvic acid at 37°C. The initial velocity of a decrease in absorbance at 340 nm due to the formation of NAD+ was measured for 3 min.

To determine the selectivity of inhibition between mono(ADP-ribosyl)ation and poly(ADP-ribosyl)ation, the effect of DR2313 on mono(ADP-ribosyl)ation of eukaryotic elongation factor 2 (EF-2) catalyzed by bacterial diphtheria toxin was examined as reported previously (Zhang et al., 2000) with some modifications. A 100-µl reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA, 0.5 mM magnesium acetate, 80 mM potassium acetate, 3 µM [32P]NAD+ (3.7–7.4 kBq), 1.0 µl of rabbit reticulocyte lysate, and 10 nM activated diphtheria toxin with various concentrations of DR2313. The reaction was performed at 25°C for 3 min and terminated with an equal volume of SDS sample buffer, which consisted of 0.25 M Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 30% glycerol, and 0.01% bromophenol blue, and the sample was heated at 100°C for 2 min. Mono(ADP-ribosyl)ated EF-2 was separated on 5% to 20% SDS-polyacrylamide gel electrophoresis (DRC, Tokyo, Japan), and the dried gels were subjected to autoradiography using a Fujix BAS-3000 (Fuji Photo Film, Tokyo, Japan).

Assay for Radical Scavenging Activity. To assess whether DR2313 has the activity of scavenging ROS, the effect of DR2313 on the hydroxyl radical-induced lipid peroxidation was examined using thiobarbituric acid reactive substances (TBARS) assay (Callaway et al., 1998). Briefly, male rat brain homogenates (10%, w/v) were prepared in ice-cold 20 mM Tris-HCl (pH 7.4) using Polytron homogenizer. Lipid peroxidation was stimulated in assays containing 100 µl of homogenate by the addition of 10 µl of 1 mM ammonium ferric sulfate, and the mixture was incubated for 30 min at 37°C. The reactions were terminated with the addition of ice-cold 20% (w/v) TCA, and the precipitated proteins were then removed by centrifugation at 10,000g for 15 min. The supernatants were heated with an equal volume of thiobarbituric acid (0.8%, w/v) at 95°C for 30 min and then cooled on ice before reading absorbance at 532 nm. PARP inhibitors or α-tocopherol (as a positive control) were dissolved in 50% dimethyl sulfoxide, and then 5 µl was added to each assay.

Preparation of Primary Cultures of Rat Cerebral Cortex and in Vitro Models of Cerebral Ischemia. Primary cultures of the cerebral cortex were prepared from fetal Wistar rats at 15 to 16 days of gestation (SLC, Yokohama, Japan) as described previously (Eliasson et al., 1997) with some modifications. Briefly, the cerebral cortex was dissected aseptically, and the blood vessels and the meninges were carefully removed under a microscope. The tissue was digested at 37°C in phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.5% glucose for 30 min. Cells harvested by centrifugation at 800g for 5 min were resuspended in DMEM supplemented with 10% fetal calf serum (SRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and seeded in 96-well culture plates (5 x 10^4/cm²) or two-well slide chambers (1 x 10^4/cm²) coated with 0.5% polyethyleneimine in 150 mM H2BO3 buffer (pH 8.4). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2. After 24 h, the medium was changed to neurobasal medium supplemented with B27 and the antibiotics. The cultures were used at 7 to 10 days after plating. The cells were washed twice with Locke’s solution containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 1.2 mM MgCl2, 2.3 mM CaCl2, 5 mM glucose, and 5 mM Hepes-KOH (pH 7.2) and incubated for 10 min at 37°C. For the induction of oxidative injuries, the cells were exposed to 500 µM hydrogen peroxide in Locke’s solutions for 4 h at 37°C. For the induction of excitotoxic injuries, the cells were exposed to 1 mM glutamate in Locke’s solution for 30 min at 37°C, washed twice with serum-free DMEM, and incubated in the same medium for 22 h. After incubations, the viability of cells was assessed with alamar blue (BioSource International, Camarillo, CA) according to the manufacturer’s protocol. The cells were preincubated with the various concentrations of DR2313 in Locke’s solution for 30 min before the induction of both ischemic injuries, and the treatments with DR2313 were kept until the end of assay.

Detection of Poly(ADP-Ribose) in Primary Cultures by Immunofluorescence. Immunofluorescence assay was performed according to a reported method (Ame et al., 1999). The cells were washed twice with PBS, fixed with ice-cold methanol/acetone (1:1) at 4°C for 10 min, and incubated with a mouse monoclonal antibody (10H, 1:400) against PAR at 4°C overnight. The specific signal of PAR was visualized by the staining of the cells with Alexa488-conjugated goat anti-mouse IgG antibody (1:2000). The nuclei were stained by propidium iodide (2 µg/ml in PBS).

Focal Cerebral Ischemia Models in Rats. The animal experimental procedures were performed in accordance with the guidelines of the Animal Ethical Committee of Pharmaceutical Research Center at Meiji Seika Kaisha Ltd. The animals were maintained on ordinary laboratory chow and tap water ad libitum under a constant 12-h light/dark cycle. Focal cerebral ischemia in rats was produced by a modification of the intraluminal thread method described previously (Longa et al., 1989). Male Wistar rats (SLC) weighing 220 to 300 g were anesthetized with 1.0 to 1.5% halothane in 70% N2O and 30% O2 delivered through a facial mask. The rectal temperature was monitored using a rectal probe and maintained around 37°C using a thermostatically controlled heating blanket and an overhead lamp (Harvard Apparatus Inc., Holliston, MA). For continuous administration of vehicle (saline) or compounds, a polyethylene tubing (PE-50; BD Biosciences, San Jose, CA) was inserted into the right jugular vein and externalized at the dorsal neck. Under an operating microscope, a 3-0 nylon monofilament (Niko Kogyo, Tokyo, Japan), with its tip being rounded by heat, was inserted into the left internal carotid artery through the left external carotid artery and advanced approximately 18 to 18.5 mm intracranially from the common carotid artery bifurcation to occlude the origin of left middle cerebral artery (MCA). After the neck incision was closed, the halothane anesthesia was ceased, and the rats were returned to their home cages. They awoke 5 to 10 min after termination of anesthesia, and the neurological deficits were evaluated as described previously (Longa et al., 1989). The rats satisfying some criteria of neurological deficits were selected and examined for the following studies. For permanent MCA occlusions (pMCAo), the monofilament was kept in place for 24 h. For transient MCA occlusions (tMCAo), the rats were reanesthetized, and reperfusion was performed by the withdrawal of the monofilament until the tip cleared from the internal carotid artery lumen and reached the origin of external carotid artery. The incision was reclosed, and the rats were returned to their home cages.

Compound Administration (Study Design). DR2313 was dissolved in saline (3 or 10 mg/ml). MCI-186 was dissolved in 1 N NaOH and adjusted to pH 7.4 with 1 N HCl to prepare the solution of 30 mg/ml and diluted to 3 mg/ml with saline (Watanabe et al., 1994). DR2313 or MCI-186 was administered via the right jugular vein as a slow bolus injection (1 ml/kg) given by hand, followed by a continuous infusion (1 ml/kg/h) with an infusion pump (Harvard Apparatus). For the study of pMCAo, DR2313 was injected as a bolus 5 min before ischemia and then infused for 6 h (Fig. 6, experiment 1). For the study of tMCAo (1.5 h of ischemia), DR2313 was injected as two boluses (5 min before ischemia and 5 min before reperfusion) and infused from the first bolus injection up to 6 h (Fig. 6, experiment 2).
Therapeutic time window of the neuroprotection by DR2313 or MCI-186 was evaluated in the tMCAo model (1 h of ischemia) by systemically delaying the initiation of each administration (Fig. 6, experiment 3). In this study, pretreatment of each compound was performed by the same procedure as described in the experiment 2. The efficacy of post-treatment was examined by a bolus at the initiation of each administration followed by infusion for 6 h.

**Determination of Infracnt Vulfme.** The infract volume was measured by 2,3,5-triphenyltetrazolium chloride staining. At 24 h after MCA occlusions, rats were reanesthetized with pentobarbital sodium (50 mg/kg i.p.) and decapitated. The brains were immediately removed into ice-cold saline and sectioned into the six coronal slices at 2-mm intervals using a brain matrix (Neurosearch, Tokyo, Japan) on ice. The brain slices were incubated in saline containing 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 min with gentle shaking and fixed in 10% buffered formalin solution for 48 h. The damaged volume was measured at each sectional level using a computer-based image analyzer (NIH Image, version 1.61). The total volume of infarction was calculated by the summation of the damaged volume in each brain slice. Alternatively, the slices (coronal section 3) were embedded in paraffin and the thin sections (2 μm) at +1.7 mm from Bregma were stained with hematoxylin-eosin.

**Ex Vivo PARP Assay.** The naive rats were given DR2313 (a bolus of 3 or 10 mg/kg and infusion at 3 or 10 mg/kg/h) as described above and sacrificed by decapitation at intervals. The whole brain was immediately removed and stored at −80°C until analysis. Frozen brains were thawed, weighed, and homogenized by a sonicator (Branson Sonifier-250) on ice in two volumes of buffer A. The ex vivo PARP assays were started by a direct addition of 1 μl of 25 mM NAD+ containing [32P]NAD+ to the mixtures were incubated for 30 min at 37°C. The reactions were terminated with the addition of 0.2% (w/v) TCA, and the rest of the procedures were the same as in the PARP inhibition assay.

**Measurements of DR2313 Levels in Rat Brain.** The concentration of DR2313 in the rat brain was determined by reverse-phase high-performance liquid chromatography (RP-HPLC)/UV system. DR2313 was administrated to the rats as described in ex vivo PARP assay, and the whole brain removed at intervals was homogenized in 3 volumes of ice-cold 0.6 M perchloric acid. The brain extracts were directly injected onto the RP-HPLC system. A LC-6A pump (Shi-zen) was used for the elution of DR2313. The mobile phase consisted of 0.1 M potassium phosphate buffer (pH 6.5) and 7% acetonitrile. Separation of DR2313 was performed isocratically at room temperature and at a flow rate of 1.0 ml/min. In these analytical conditions, DR2313 was eluted at 8 min.

**Measurement of Physiological Parameters.** The naive rats were anesthetized with halothane (induction, 4.0%; maintenance, 1.0–1.5%) in 70% N2O and 30% O2 delivered through a facial mask. The rectal temperature was measured using a rectal probe. The right femoral artery was cannulated with PE-50 tubing for a continuous monitoring of the mean arterial blood pressure (MABP) and heart rate (HR) in the polygraph system (RM-6000; Nihon Kohden, Tokyo, Japan). The left common carotid artery was also cannulated for the measurement of the blood parameters (pH, pO2, pCO2, and hematocrit) in the 288 Blood Gas system (Ciba Corning Diag., Medford, MA).

**Statistical Analysis.** All data are represented as the mean ± S.E. The IC50 and the ED50 values from studies in vitro were calculated using KaleidaGraph 3.0.2. software (Abelbeck software). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple-range test for drug-treated groups versus control (vehicle-treated) group. P values less than 0.05 were considered to be significant.

**Results**

**Structure and Solubility of DR2313.** Figure 1 showed the chemical structure of DR2313 (C8H12NO5S, MW 182.24). DR2313 does not contain the moiety of benzamide, whereas the previously reported PARP inhibitors, 3AB, PND, and isooquinolinone derivatives (DIQ and DPQ) contain benzamide (indicated by bold lines). DR2313 and 3AB were readily dissolved in aqueous vehicle such as saline or water at 30 and 14 mg/ml, respectively (data not shown). On the other hand, all other compounds were insoluble in water, even at 1 mg/ml (data not shown).

**PARP Inhibitory Activity of DR2313.** DR2313 showed more powerful inhibition of the poly(ADP-ribo-sylation) in the nuclear extracts of the rat brain (IC50 value, 0.20 ± 0.01 μM) than 3AB (35.4 ± 3.79 μM), PND (0.56 ± 0.02 μM), DIQ (2.96 ± 0.19 μM), and DPQ (0.96 ± 0.04 μM) (Fig. 2A). The enzyme inhibition mode was analyzed by Dixon’s plot. DR2313 inhibited the poly(ADP-ribo-sylation) reaction in a typical competitive manner (Fig. 2B). The Ki value for DR2313 was determined to be 0.23 μM, which was almost identical to the calculated IC50 value; thus, the plot indicates that DR2313 competed with NAD+ at the PARP catalytic site.

**Selectivity and Radical Scavenging Activity of DR2313.** The selectivity of DR2313 to PARP-1 and PARP-2 was evaluated using each purified recombinant enzyme (Fig. 3A). DR2313 showed almost identical inhibition to both PARP-1 (IC50 value, 0.20 μM) and PARP-2 (IC50 value, 0.24 μM). Next, to determine the selectivity of inhibition between mono(ADP-ribo-sylation) and poly(ADP-ribo-sylation), the effects of DR2313 on mono(ADP-ribo-sylation) of eukaryotic EF-2 catalyzed by bacterial diphtheria toxin was investigated (Fig. 3A). DR2313 showed a weak inhibition of the mono(ADP-ribo-sylation) in a concentration-dependent manner (IC50 value, 59 μM). Furthermore, DR2313 had no effect on GAPDH, ADH, or LDH activity at a concentration of 10 μM but slightly inhibited ADH and LDH activity at a con-
Fig. 2. PARP inhibitory activity and enzyme kinetic analysis of PARP inhibition by DR2313. A, PARP inhibitory activity of DR2313 (○) in the nuclear extracts of the rat brain compared with that of PND (●), DPQ (△), DIQ (□), or 3AB (■). IC_{50} values were calculated from the concentration dependence of the inhibition curves using nonlinear regression analyses assisted by KaleidaGraph 3.02 software. Data (percentage of control) are indicated as the mean ± S.E. of eight samples from four independent experiments. B, Dixon’s plot analysis shows that the inhibitory action of DR2313 is competitive, and the $K_i$ value is estimated to be 0.23 μM. NAD$^+$ at the three concentrations (○), 125; □, 250; and ■, 500 μM were incubated in the reactions with or without DR2313 (0.016–16.4 μM). Data (1/V: minutes per milligram of protein per picomole of NAD$^+$) are indicated as the mean ± S.E. of six samples from three independent experiments.

Neuroprotective Effects of DR2313 in in Vivo Models of Cerebral Ischemia. To determine neuroprotective effects of DR2313 in vivo, experiments of pMCAos and tMCAos in rats were conducted with the administration of DR2313 beginning 5 min before the onset of ischemia (Fig. 6, experiments 1 and 2).

pMCAo Model. In a vehicle-treated group, pMCAo induced the extensive damage in both the cortex and noncortex regions (Fig. 7C), with the volume of infarction being 190.7 ± 13.0 and 86.6 ± 8.4 mm$^3$, respectively (Fig. 7A). Pretreatment with DR2313 reduced the infarct volume in a dose-dependent manner, and in a high dose-treated group (10 mg/kg i.v. bolus and 10 mg/kg/h i.v. infusion for 6 h), a reduction in the total and cortical infarct volume reached statistical significance [percentage inhibition: 33% (P = 0.029) and 38% (P = 0.014), respectively] compared with that in the vehicle-treated group (Fig. 7A).

tMCAo Model. In a vehicle-treated group, tMCAo (1.5 h of ischemia) induced less severe damage in the cortex (150.3 ± 12.8 mm$^3$) and noncortex (59.1 ± 4.6 mm$^3$) regions, compared with that of pMCAo (Fig. 7B). Again, pretreatment with DR2313 reduced the infarct volume dose dependently, and in a high dose-treated group, the total and cortical infarct volume was reduced significantly [percentage inhibition: 49% (P = 0.034) and 50% (P = 0.033), respectively] compared with the vehicle-treated group (Fig. 7B).

Therapeutic Time Window of the Neuroprotection by DR2313. To evaluate the therapeutic time window of the neuroprotection by DR2313, the effect of post-treatment with DR2313 in tMCAo model (1 h of ischemia) was examined and compared with that of a free radical scavenger, MCI-186 (Fig. 6, experiment 3). The dosing regimen of MCI-186 was selected according to Watanabe et al. (1994) and our preliminary experiments. Pretreatment with DR2313 significantly reduced the infarct volume (percentage inhibition: 62%) compared with the vehicle-treated group (P = 0.003; Fig. 8A). Post-treatment with DR2313 delaying for 2 h after ischemia exerted a significant reduction in the infarct volume (percentage inhibition: 47%, P = 0.041; Fig. 8A). In 4 h post-treatment, the infarct volume was similar to that in 2 h...
post-treatment (percentage inhibition: 44%), but this did not reach statistical significance ($P = 0.056$; Fig. 8A). In 6 h post-treatment, there was no effect of DR2313 on the infarct volume. Pretreatment with MCI-186 also reduced the infarct volume (percentage inhibition: 59%), and the efficacy was at the same level of DR2313 ($P = 0.006$; Fig. 8B). However, the neuroprotective effect of MCI-186 disappeared when the start of treatment was delayed for 1 or 2 h, which was unlike DR2313 (Fig. 8B).

**Physiological Parameters.** There were no significant differences in the MABP (Fig. 9A), HR (Fig. 9B), and rectal temperature (Fig. 9C) in the vehicle- or DR2313-treated groups in naive rats. There were also no significant differences in arterial blood parameters ($\text{pH}$, $\text{pO}_2$, $\text{pCO}_2$, and hematocrit) in the vehicle- and DR2313-treated groups (data not shown).

**Discussion**

In the present study, we first evaluated the pharmacological profiles of a newly synthesized PARP inhibitor, DR2313. This compound showed more powerful inhibition of poly-(ADP-ribosyl)ation in nuclear extracts of the rat brain than the reported PARP inhibitors (Fig. 2A). In addition, the analysis of enzymatic kinetics demonstrated that DR2313 was a competitive type of inhibitor (Fig. 2B). Most of the PARP inhibitors reported so far are structural analogs of $\text{NAD}^+$ and are thought to compete with $\text{NAD}^+$ itself at the catalytic domain (Costantino et al., 2001). A crystallographic analysis...
of the catalytic fragment of PARP-1 with the reported inhibitors has suggested that the moiety of benzamide is a pharmacophore for inhibiting poly(ADP-ribosyl)ation (Costantino et al., 2001). Namely, the amide moiety invariably interacts with the backbone atoms of Gly863, whereas the aromatic portion (benzene ring) interacts, presumably through a π-π interaction, with Tyr907 (Costantino et al., 2001; Ferraris et al., 2003). Thus, most of the PARP inhibitors contain the moiety of benzamide (Fig. 1). In contrast, DR2313 possesses the amide moiety, which is thought to interact with Gly863, but does not contain the benzene ring. The interaction of DR2313 with Tyr907 is probably via the dihydro-thiopyran ring because aliphatic rings such as a pyran can interact with tyrosine by their hydrophobicities. This prediction is also supported by our computational analysis based on the crystallographic information of PARP-1 (unpublished data). This novel structure of DR2313, so-called nonbenzamide one, may exhibit unique physiochemical and/or pharmacological characteristics. In fact, the calculated LogP value of DR2313 was −2.2, which suggests favorable solubility to aqueous solvents, whereas the calculated LogP values of PND, DIQ, and DPQ were over +2.0 (data not shown). Thus, DR2313 is a more potent and water-soluble PARP inhibitor than the reported compounds.

Recently, new members of the PARP family have been identified. The PARP-1 homology domain containing the NAD⁺-binding site is highly conserved among all of the members (Chiarugi, 2002). Therefore, 3AB, which is often used as the PARP-1 inhibitor, can also inhibit PARP-2 (Ame et al., 1999). Similarly, DR2313 could equally inhibit both PARP-1 and PARP-2 (Fig. 3A). Some of the reported PARP inhibitors have also potencies to inhibit other NAD⁺-utilizing enzymes, because of their homology at the catalytic domain (Banasik et al., 1992; Zhang et al., 2000). In particular, amino acid sequences at the NAD⁺-binding site between bacterial mono-(ADP-ribosyl)transferase (e.g., diphtheria toxins) and PARP-1 have been remarkably conserved (Marsischky et al., 1995). Regardless of their homology, DR2313 is more selective to poly(ADP-ribosyl)ation by PARP-1 (IC₅₀ value, 0.2 μM) than to mono(ADP-ribosyl)ation by diphtheria toxins (IC₅₀ value, 59 μM) (Fig. 3A). Furthermore, DR2313 had few effects on the reactions exchanging NAD⁺ to NADH by several dehydrogenases (Fig. 3A). These results suggest that DR2313 could be a useful agent to inhibit PARP more selec-
tively than other NAD\textsuperscript{+}-utilizing enzymes but could not recognize the difference among PARP isozymes. In cultured neuron, oxidative stress triggered by ROS mediates glutamate neurotoxicity and induces various types of cell damage, which are partially attributed to over-activation of PARP-1 (Eliasson et al., 1997; Pieper et al., 2000). The present study demonstrated that the exposure of rat primary cultures to hydrogen peroxide or glutamate resulted in severe cell death, and hydrogen peroxide led to the massive formation of PAR in the nucleus (Fig. 4). Pretreatment with DR2313 significantly reduced these cell deaths and completely inhibited the hydrogen peroxide-induced formation of PAR (Fig. 4). As shown in Fig. 3, DR2313 did not possess the hydroxyl radical-scavenging activity in vitro. Also, this com-

Fig. 7. Neuroprotective effects of DR2313 in in vivo models of cerebral ischemia. Neuroprotective effects of DR2313 on the infarct volume induced by pMCAo (A) or tMCAo (B) in rats. DR2313 was administered as shown in experiment 1 or 2 from Fig. 6. Open, hatched, or closed columns indicate vehicle (saline), the low dose (3 mg/kg i.v. bolus and 3 mg/kg/h i.v. infusion for 6 h), or the high dose (10 mg/kg i.v. bolus and 10 mg/kg/h i.v. infusion for 6 h)-treated group, respectively. The infarct volume was indicated as total, cortex, and noncortex regions (total-minus-cortex regions; almost identical to the regions of striatum). Data (millimeters cubed) are indicated as the mean ± S.E., \( P < 0.05 \) (ANOVA followed by Dunnett’s multiple range test) compared with vehicle-treated group. The number of animals used in each group is shown in inset. Histochemical examinations by hematoxylin-eosin staining were performed (C). The thin sections (2 \( \mu \)m) at +1.7 mm from bregma were evaluated. a and c are representative for the staining in vehicle-treated group, b and d are representative for the staining in high dose-treated group of DR2313. C or NC in each section, cortex or noncortex region, respectively. Scale bars are 2 mm.

Fig. 8. Therapeutic time window of the neuroprotection by DR2313 or MCI-186. Therapeutic time window of the neuroprotection by DR2313 (A) or MCI-186 (B) in tMCAo (1 h ischemia) in rats was evaluated. Each compound was administered as shown in experiment 3 from Fig. 6. The values were determined at 30 min before the administrations and 10, 30, 60, 120, 240, 360, or 480 min after the administrations. Data are indicated as the mean ± S.E. of three to four animals.

Fig. 9. Effects of DR2313 on MABP, HR, and rectal temperature in naive rats under anesthesia. The naive rats were anesthetized with halothane (induction, 4.0%; maintenance, 1.0–1.5%) in 70% \( \text{N}_2\text{O} \) and 30% \( \text{O}_2 \) delivered through a facial mask. The right femoral artery was cannulated with a PE-50 tubing for continuous monitoring of MABP (A) and HR (B). The rectal temperature (C) was monitored using a rectal probe. DR2313 (low dose-treated group, \( \Delta \); high dose-treated group, \( \square \)) or vehicle (○) was administered as shown in experiment 1 from Fig. 6. The values were determined at 30 min before the administrations and 10, 30, 60, 120, 240, 360, or 480 min after the administrations. Data are indicated as the mean ± S.E. of three to four animals.
ound has no significant affinities to the NMDA receptor (glutamate binding site) and nNOS, which have been implicated in the glutamate-induced neurotoxicity (data not shown). Moreover, the neuroprotective concentrations (ED50) of DR2313 in both hydrogen peroxide and glutamate toxicity assays were almost identical to its PARP inhibitory activity (IC50) in an enzyme assay (Figs. 2 and 4). Taken together, these results suggest that DR2313 might protect neurons from ROS-mediated neurotoxicity via its PARP inhibition in vitro.

To clearly define the role of PARP in cerebral ischemia, a specific PARP inhibitor that readily penetrates the blood brain barrier is required. Several reported inhibitors including 3AB do not easily cross the barrier (Andersson et al., 1994). The ex vivo PARP assay provided a convenient system to measure the brain penetrability of DR2313. The present data (Fig. 5) demonstrated that DR2313 could rapidly penetrate the barrier and dose-dependently inhibit the brain PARP activity by i.v. injection. This pharmacodynamic analysis appeared to correlate quite well with the pharmacokinetic data (Fig. 5). It is important to note that the brain samples for the ex vivo assay were diluted by 2-fold, indicating that the inhibitory activities of DR2313 in the rat brain might be more strong than actually measured levels. Thus, DR2313 can readily penetrate the blood brain barrier with the peripheral administration, and this dosing regimen proves to be quite optimal for inhibiting the brain PARP activity.

The present study demonstrated that the i.v. treatment with DR2313 (bolus and infusion) before ischemia reduced the infarct volume dose dependently in both permanent and transient MCAo models in rats. The maximal reduction of infarct volume in these models was obtained by 33 and 49%, respectively, in each high dose-treated group (Fig. 7). Other PARP inhibitors, such as DPQ and 3AB, also provided the equal degree of the neuroprotection in the same conditions, but the routes of administration were often intraperitoneal or intraventricular because of the insolubility to aqueous vehicle or the inability to penetrate the blood brain barrier (Takahashi et al., 1997; Tokime et al., 1998). For the treatment of acute ischemic stroke, a compound should ideally be soluble in aqueous vehicle and deliverable by bolus injection or infusion. Thus, the experimental protocol used here may be comparably adequate for treating ischemic stroke in a clinical trial. Recently, PJ34, a new class of potent PARP inhibitor, was synthesized, and the i.v. treatment demonstrated the profound reduction of infarction in the tMCAo model (Abdelkarim et al., 2001), unlike DR2313. The differences observed between these inhibitors may be related to the dosing regimen, the bioavailability, or unknown mechanisms of action, rather than the potency, because another new PARP inhibitor, INO-1001, has been also reported to reduce the infarction in pMCAo model (Komiji et al., 2004).

In focal cerebral ischemia, ROS have been implicated in the development of brain damage (Chan, 2001). ROS formation is increased after permanent and transient MCAo model in rats (Peters et al., 1998). Thus, it has been demonstrated that a compound with ROS scavenging activity has the ability to attenuate this cellular damage in stroke (Watanabe et al., 1994; Kawai et al., 1997). In the present study, DR2313 did not show the radical-scavenging activity in vitro, whereas 3AB and DIQ significantly reduced the formation of TBARS (Fig. 3B), indicating a possibility that not only the inhibition of brain PARP activity but also the radical scavenging action might contribute to the neuroprotective efficacies of these reported inhibitors, as described in other reports (Tokime et al., 1998; Chiarugi et al., 2003). Therefore, the reduction of infarct volume by the treatment with DR2313 might reflect the effect obtained by the specific inhibition of brain PARP activity in focal cerebral ischemia.

MCI-186 (edaravone) is the first neuroprotectant used clinically in Japan for the treatment of stroke patients within 24 h after the onset of ischemia (Tanaka, 2002). In a preclinical study, the neuroprotective effects of MCI-186 were also confirmed in a rat global (Watanabe et al., 1994) and focal ischemia model (Kawai et al., 1997). To predict the clinical efficacy of DR2313 in stroke patients, therefore, it is quite important to compare the neuroprotective effect of DR2313 with that of MCI-186 in animal models. We found that the pretreatment with MCI-186 reduced the infarction in the tMCAo model, and the degree of neuroprotective effect was almost similar to that of DR2313 (Fig. 8). To our knowledge, this is the first study in which the neuroprotective effect of a PARP inhibitor was compared with that of a radical scavenger in rat focal cerebral ischemia. In addition, the post-treatment with DR2313 delaying for 2 h after ischemia significantly prevented the progression of infarction, and the 4 h post-treatment exerted a similar reduction of infarct volume to that in the 2 h post-treatment (Fig. 8). On the other hand, the post-treatment with MCI-186 failed to show any neuroprotective effects, even by the 1 h post-treatment after ischemia (Fig. 8). The time between the onset of ischemia and the initiation of therapy is a critical factor in treating acute ischemic stroke. Pharmacologically targeting a downstream event in the cerebral ischemic cascade, like overactivation of PARP-1, could theoretically prolong the time for initiation of therapy (Markgraf et al., 1998; Takahashi et al., 1999). In fact, our results obtained by the study of therapeutic time window (Fig. 8) support this idea. Moreover, DR2313 did not show any significant effects on the physiological parameters in naive rats (Fig. 9). Therefore, the therapeutic treatment with DR2313 may be well tolerated and more efficacious in treating acute ischemic stroke than that with free radical scavenger.

In conclusion, DR2313, a potent PARP inhibitor, could be a promising drug for stroke therapy by virtue of the excellent water solubility, brain penetrability, and neuroprotective efficacy.

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References

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