A New Poly(ADP-Ribose) Polymerase Inhibitor, FR261529 [2-(4-Chlorophenyl)-5-quinoxalinecarboxamide], Ameliorates Methamphetamine-Induced Dopaminergic Neurotoxicity in Mice

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ABSTRACT

Methamphetamine (METH) administration in mice, results in a chronic dopamine (DA) depletion associated with nerve terminal damage, with DA oxidation and generation of reactive oxygen species (ROS) primarily mediating this neurotoxicity. The oxidative stress induced by METH putatively activates nuclear enzyme poly(ADP-ribose) polymerase (PARP), with excessive PARP activation eventually leading to cell death. In this study, we show that prevention of PARP activation by treatment with FR261529 [2-(4-chlorophenyl)-5-quinoxalinecarboxamide], the compound that was recently identified as a novel PARP inhibitor (IC50 for PARP-1 33 nM, IC50 for PARP-2 = 7 nM), protects against both ROS-induced cell injury in vitro and METH-induced dopaminergic neuronal damage in an in vivo Parkinson’s disease (PD) model. In PC12 cells, exposure of hydrogen peroxide or METH markedly induced PARP activation, and treatment with FR261529 (1 μM) significantly reduced PARP activation and attenuated cell death. In the mouse METH model, METH (15 mg/kg, 2 h apart) intoxication accelerated DA metabolism and oxidation in the striatum, with subsequent cell damage in nigrostriatal dopaminergic neurons after 4 days. Oral administration of FR261529 (10 or 32 mg/kg) attenuated the damage of dopaminergic neurons via marked reduction of PARP activity and not via changes in dopamine metabolism or body temperature. These findings indicate that the neuroprotective effects of a novel PARP inhibitor, FR261529, were accompanied by inhibition of METH-induced PARP activation, suggesting that METH induces nigrostriatal dopaminergic neurodegeneration involving PARP activation and also orally active and brain-penetrable PARP inhibitor FR261529 could be a novel attractive therapeutic candidate for neurodegenerative disorders such as PD.

Poly(ADP-ribose) polymerase (PARP) is activated after DNA damage that mediates neuronal cell death, providing a mechanism by which PARP inhibitors can exert neuroprotection. Reactive free radical species-mediated damage of DNA can activate PARP (Szabo et al., 1996; Eliasson et al., 1997) and consumes NAD and consequently ATP, culminating in cell dysfunction or necrosis (Ha and Snyder, 1999). PARP also plays a central role in a caspase-independent apoptosis pathway mediated by apoptosis-inducing factor (Yu et al., 2002). Translocation of plays a central role in a caspase-independent apoptosis pathway mediated by apoptosis from the mitochondria to the nucleus is dependent on PARP activation in neurons treated with various DNA-damaging stimuli such as N-methyl-D-aspartate (NMDA) or hydrogen peroxide (Yu et al., 2002). This cellular suicide mechanism of both necrosis and apoptosis by PARP activation has been implicated in the pathogenesis of brain injury and neurodegenerative disorders, and PARP inhibitors have been shown to be effective in animal models of stroke (Takahashi et al., 1999; Abdelkarim et al., 2001; Iwashita et al., 2004a), traumatic brain injury (LaPlaca et al., 2001), and Parkinson’s disease (PD; Cosi et al., 1996; Iwashita et al., 2004b).

Methamphetamine (METH) intoxication in mice produces neurodegeneration of dopaminergic terminals in the striatum and cell body loss in the substantia nigra pars compacta (SNpc) (Sonsalla et al., 1996; Hirata and Cadet, 1997; Kita et al., 1998). METH enters nerve terminals via the dopamine transporter (DAT), dopamine transporter; DA, dopamine; MAO, monoamine oxidase; ROS, reactive oxygen species; nNOS, neuronal nitric-oxide synthases; FR261529, 2-(4-chlorophenyl)-5-quinoxalinecarboxamide; 3-AB, 3-aminobenzamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; TCA, trichloroacetic acid; KRH, Krebs-Ringer-HEPES; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; NOS, nitric-oxide synthase; LDH, lactate dehydrogenase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 3-MT, 3-methoxytyramine; ANOVA, analysis of variance; TH, tyrosine hydroxylase.
transporter (DAT) and displaces both vesicular and intracellular dopamine (DA). This displacement results in increased DA release within the cytoplasm of DA terminals, with subsequent DA oxidation by monoamine oxidase (MAO) activity and auto-oxidation to reactive oxygen species (ROS) and H$_2$O$_2$ (Cubells et al., 1994; Cadet and Brannock, 1998). The role of oxidative stress in the neurochemical actions of METH is supported by the fact that METH can cause lipid and protein oxidation in mouse brain (Jayanthi et al., 1998; Gluck et al., 2001) and that the administration of antioxidants, such as ascorbate and vitamin E, can attenuate METH-induced toxicity (De Vito and Wagner, 1989). Transgenic mice overexpressing the antioxidant enzyme copper/zinc superoxide dismutase showed an attenuated response to neurotoxic doses of METH (Cadet et al., 1994).

METH administration can also lead to increased levels of extracellular glutamate (Nash and Yamamoto, 1992), leading to excessive NMDA receptor activation and formation of superoxide and nitric oxide (Lafon-Cazal et al., 1993; Gunasekar et al., 1995). Superoxide reacts with nitric oxide to produce peroxynitrite, a highly cytotoxic compound, that causes neuronal death in numerous models of brain damage, including cerebral ischemia and PD (Tabner et al., 2001).

Pharmacological inhibition of various neuronal nitric-oxide synthases (nNOS) reduces METH-induced neurotoxicity, and mice lacking the nNOS gene are resistant to this neurotoxicity (Di Monte et al., 1996; Itzhak et al., 1998).

Changes in body temperature can also markedly influence the degree of METH neurotoxicity, with higher temperatures causing greater toxicity and lower temperatures affording neuroprotection (Bowyer et al., 1992; Albers and Sonsalla, 1995). Furthermore, severe hyperthermia may result in the activation or up-regulation of proteins capable of oxidizing DA and formation of DA quinine (LaVoie and Hastings, 1999). This evidence suggests that increased DA oxidation, generation of reactive species such as superoxide or peroxynitrite, as well as hyperthermia, are mediators of METH neurotoxicity.

Direct evidence for the involvement of PARP in the pathogenesis of METH-induced neurotoxicity comes from the evaluation of PARP inhibitors in mice. METH intoxication significantly reduced the levels of striatal dopamine in C57BL/6 mice, whereas treatment with the conventional PARP inhibitor benzamide ameliorated the depletion induced by METH (Così et al., 1996). This led us to hypothesize that METH intoxication might induce nigrostriatal dopaminergic neurodegeneration in mice via PARP activation downstream of ROS generation and that PARP inhibition could ameliorate METH-induced neurotoxicity.

We have recently identified FR261529 [2-(4-chlorophenyl)-5-quinoxalinecarboxamide; chemical structure shown in Fig. 1] as a novel and potent PARP inhibitor, by applying the rational discovery strategies, such as structure-based drug design, combinatorial chemistry, and conventional structure-activity relationship for improvement of potency. The purpose of the present study was, first, to investigate the PARP-inhibiting activity and specificity of FR261529. The second purpose was to determine the PARP inhibitory properties and the neuroprotective properties of FR261529 in two in vitro experimental neuronal cell death models, in which PARP is markedly activated by H$_2$O$_2$ or METH exposure. Finally, the neuroprotective properties of FR261529 and the effect of FR261529 on DA release, lipid peroxidation, and hyperthermia were evaluated in a mouse METH model, a relevant in vivo model of PD. 3-Aminobenzamide (3-AB) was also evaluated in this study as a reference PARP inhibitor.

**Materials and Methods**

**Materials**

Rat phaeochromocytoma PC12 cells were purchased from American Type Culture Collection (Manassas, VA). FR261529 (chemical structure shown in Fig. 1) was synthesized at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). 3-AB was purchased from Sigma-Aldrich (St. Louis, MO). METH (methamphetamine hydrochloride) was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Hydrogen peroxide (30%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were from Wako Pure Chemicals (Tokyo, Japan). Hydrogen peroxide (30%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were from Wako Pure Chemicals (Tokyo, Japan). Recombinant human PARP enzyme was purchased from Trevigen (Gaithersburg, MD), and recombinant mouse PARP-2 enzyme was purchased from Alexis Biochemicals (San Diego, CA). Unless otherwise stated, all other materials were purchased from Sigma-Aldrich.

**Measurement of PARP Inhibitory Activity in Vitro**

To assess the PARP-1 or PARP-2 inhibitory activity of FR261529 and 3-AB, PARP activity was evaluated as described previously (Banasik et al., 1992) with minor modifications. PARP enzyme assay was carried out in a final volume of 100 μl consisting of 50 mM Tris-HCl (pH 8.0), 25 mM MgCl$_2$, 1 mM dithiothreitol, 10 μg of activated salmon testes DNA, 0.1 μCi of [adenylate-$^{32}$P]NAD, 0.2 units of recombinant human PARP for PARP-1 assay or 0.1 units of recombinant mouse PARP-2 for PARP-2 assay, and various concentrations of FR261529 or 3-AB. The reaction mixture was incubated at room temperature (23°C) for 15 min, and the reaction was terminated by adding 200 μl of ice-cold 20% trichloroacetic acid (TCA) and incubated at 4°C for 10 min. The precipitate was transferred onto GF/B filter (Packard Unifilter-GF/B) and washed three times with 10% TCA solution and 70% ethanol. After the filter was dried, the radioactivity was determined by liquid scintillation counting.

**Preparation of Nuclear Extracts from PC12 Cells and the Mouse Brain**

Published methods were used for preparation of nuclear extracts, with minor modifications (Lahiri and Ge, 2000). To prepare the nuclear extracts from PC12 cells, 2 × 10$^6$ cells cultured in 25 cm$^2$ flask were washed with 10 ml of phosphate-buffered saline, and cells were resuspended in 500 μl of cold buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM MgCl$_2$, 0.1 mM EDTA, and 0.1% Nodidet-40) and homogenized gently. The homogenate was centrifuged at 5000 × g for 30 s, and the supernatant containing cytoplasm and RNA was removed. The nuclear pellet was resuspended in 50 μl of ice-cold buffer B (50 mM HEPES, pH 7.9, 400 mM KCl, 0.1 mM EDTA, and 10% glycerol). The tube was mixed thoroughly and placed on a microtube mixer for 15 min at 4°C.

![Fig. 1. Chemical structure of FR261529.](image)
The nuclear extract was centrifuged at 11,000g for 10 min. The supernatant containing the proteins from the nuclear extract was removed carefully to a fresh tube. The protein was measured in the nuclear extract and then used for the PARP assay immediately.

For preparation of nuclear extracts from mouse brain, normal and/or drug-treated whole brains were dissected and transferred to a Teflon homogenizer. Buffer A was added at 300 mg of brain tissue per 1 ml and 10 strokes of homogenization were performed. The whole suspension was transferred equally to Eppendorf tubes followed by centrifugation in a microcentrifuge for 1 min. The supernatant containing mostly cytoplasmic constituents were removed, and 300 μl of buffer B was added to the nuclear pellet in each of the Eppendorf tubes. The tubes were mixed thoroughly and placed on a microtube mixer for 15 min. The supernatant containing the proteins from the nuclear extract was removed carefully to a fresh tube. The protein was measured in the nuclear extract and then the crude solution containing PARP enzyme and DNA was used for the PARP assay immediately.

**Specificity of FR261529**

**DAT Binding Assay.** To determine the affinity of FR261529 and 3-AB to the DAT and also to evaluate the level of cell damage after METH intoxication in mice, the striatum and/or substantia nigra were dissected from mouse brains. Homogenate with KRH buffer containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.2 mM KH2PO4, 5.6 mM glucose, 0.01 mM sodium azide, and 25 mM HEPES, pH 7.4, was prepared by several centrifugations. For the DAT binding assay, mazindol binding to the DAT was evaluated according to the method of Javitch et al. (1984) with minor modifications. Binding was assayed by addition of membrane preparations from the striatum or substantia nigra, about 125 μg of protein, to an incubation solution (KRH buffer) containing 5 nM [3H]mazindol (17 Ci/mmole; PerkinElmer Life and Analytical Sciences, Boston, MA) and 300 nM desipramine to occlude binding to the norepinephrine transporter. Samples in duplicate were incubated at 4°C for 1 h, and the reaction was stopped by addition of ice-cold KRH buffer. The reaction solution was rapidly filtered through Whatman GF/C filters and three washes with binding buffer using a cell harvester. Nonspecific binding was determined in the presence of 10 μM cocaine.

**Determination of [3H]Spiperone Binding Activity.** The affinity of FR261529 and 3-AB to dopamine D2 receptor was measured by using a [3H]spiperone binding assay as described previously (Zahniser and Dubocovich, 1983) with minor modifications. Briefly, the mice striatal membrane isolates (final 1 mg/ml) were incubated with 50 mM Tris-HCl buffer (pH 7.4), containing 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 1 mM different concentrations of drug and 3 nM [3H]spiperone (specific activity 107 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) and 300 nM desipramine to occlude binding to the norepinephrine transporter. The aliquots of supernatant were then added to an equal volume of trichloroacetic acid (TCA). The samples were heated at 95°C for 30 min and then cooled on ice before reading absorbance at 532 nm. Concentrations of TBARS were calculated using standard curve obtained with malondialdehyde (MDA). Percentage of inhibition of TBARS production was calculated as follows: % inhibition = [(max – drug)/(max – min)] × 100, where max represents values in the presence of ammonium ferric sulfate, base represents values in the absence of ammonium ferric sulfate, and drug represents values of test compounds.

**Neuroprotective Efficacy in PC12 Cells**

**Determination of PARP Activation.** To determine PARP activation directly in PC12 cells, PARP enzyme assay and NAD assay were used. PC12 cells were seeded at 2 × 106 cells in a 24-well culture flask and cultured for 24 h. Then, FR261529 was added to culture media at several concentrations. Thirty minutes later, cells were exposed H2O2 (0.1–1 mM) or METH (0.1–1 mM) for 30 min, and cells were detached using cell scraper and then nuclear extracts were prepared as described above. PARP activation was determined by PARP enzyme assay using nuclear extracts as a mixture of PARP enzyme and nicked DNA. To determine NAD level, detached cells were collected in microcentrifuge tube by centrifugation for 5 min × 100g at 4°C. Cell homogenate was extracted with 200 μl of 0.5 M HClO4 for 15 min and then 60 μl of 2 M KOH/0.2 M K2HPO4/KH2PO4 pH 7.5, was added to the acidic supernatant obtained by centrifugation. NAD level in the supernatant was measured using enzymatic conversion to NADH by alcohol dehydrogenase.

**Assessment of Cytotoxicity.** PC12 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and a 1% (v/v) penicillin-streptomycin antibiotic mixture. Cells were grown in an atmosphere of 95% air and 5% CO2 at 37°C for 24 h. For all experiments, cells were seeded at a density of 4 × 104 cells/well in 96-well culture plates and allowed to attach overnight. The cells were incubated with or without hydrogen peroxide (H2O2) and FR261529 or 3-AB for 6 h. To evaluate cell survival, MTT assay was performed according to a minor modification of the standard method. Briefly, MTT was added to the cultures at a final concentration of 0.2 mg/ml and after incubation at 37°C for 2 h, the media were removed carefully and the reaction was stopped by addition of isopropanol containing 0.04 N HCl. The absorbance of each well was measured at 590 nm using a microplate reader ( Molecular Devices, Sunnyvale, CA). In addition, hydrogen peroxide-induced cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (LDH) release with the use of the LDH assay kit (Wako Pure Chemicals).
Drug Treatment. FR261529 was dissolved in 100% dimethyl sulfoxide at 10^{-2} M and 3-AB was dissolved in phosphate-buffered saline at 100 mM, and then both were diluted in DMEM without serum. These solutions were added to culture plate 0.5 h before H_2O_2 exposure.

METH-Induced Parkinson’s Model in Mice

Animals. For METH model, 9- to 10-week-old male C57/BL6 mice (Charles River, Hino, Japan) weighing 19 to 22 g were used. All animals were housed in a room maintained at 23 ± 2°C with 55 ± 5% humidity, and with a 12-h light/dark cycle (light on at 7:00 AM). The minimum quarantine period was at least 2 week before the experiment. Animals were housed five per cage and allowed free access to food and water. On the day of the experiment, mice were maintained in a room temperature of 23°C. Rectal temperatures were recorded immediately before the first administration of drug and every hour thereafter using a small-animal rectal probe (TERUMO-CTM-303; TERUMO, Tokyo, Japan). All experiments in the present study were performed under the guidelines of the Experimental Laboratory Animal Committee of Fujisawa Pharmaceutical Co. and were in strict accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and stress to the animals during experimental procedures.

Administration of METH and PARP inhibitors. To determine the neuroprotective properties of FR261529 and 3-AB in the mouse METH model, dose finding experiments of METH intoxication were conducted. Animals received 2 × 10, 2 × 15, 2 × 25 or 4 × 15 mg/kg of METH with i.p. injection at a 2 h interval. FR261529 or 3-AB, which was suspended with 0.5% methylcellulose, was administered at doses of 3.2 to 32 mg/kg for FR261529 p.o. or 100 and 320 mg/kg for 3-AB or twice at 1h prior to first injection of METH and then 4 h later. The administration volume was adjusted to 10 ml/kg in all experiments.

Preparation of Substantia Nigra and Striatum Homogenate. For PARP assay and DAT binding assay using tissue homogenates, whole brains were dissected and put on ice-cold brain slicer immediately. One-millimeter-thick slices, including SNpc and whole bilateral striata area, were sectioned precisely by referring to mouse brain map. The exact SNpc area was also confirmed by comparison between two groups was conducted using two-tailed Student’s t test. Comparison of striatal DA, DOPAC, HVA, and 3-MT levels. The contents of DA, DOPAC, HVA, and 3-MT in the striatum were quantified using high-performance liquid chromatography with electrochemical detection. Four days after the drug administration, brains were quickly removed, and the striatum was dissected out on ice-cold dish. Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. On the day of the assay, tissue samples were homogenized by Teflon homogenizer with 0.1 M perchloric acid and 0.1 mM EDTA-2Na containing 50 ng/ml isoproterenol as internal standard. After centrifugation (15,000g, 15min, 4°C), pH of supernatant from each sample was adjusted to 3.5 with sodium acetate. Adjusted supernatants were filtered and injected onto reversed phase catecholeamine column (SC-5ODS 3.0 × 150 mm; Eicom Co., Ltd., Kyoto, Japan) with ECD-300 electrochemical detector (+450 mV). The mobile phase consisted of 0.1 M sodium acetate buffer, 0.1 M citrate buffer, pH 3.5, 0.02 mM EDTA, 1 mM sodium octane sulfonic acid, and 15% methanol. The flow rate was maintained at 0.5 ml/min. DA and its metabolites were quantified by peak height comparisons with standards run on the day of analysis. To determine whether FR261529 and other drugs affect dopamine or its metabolites content, a single dose of FR261529 (32 mg/kg p.o., nomifensine (3.2 mg/kg i.p.), haloperidol (3.2 mg/kg i.p.) or apomorphine (3.2 mg/kg i.p.) was administered, and the striatum was dissected after 1 h. The contents of DA, DOPAC, HVA, and 3-MT in the striatum were quantified as described above.

Table 1. Pharmacological characterization of 3-AB and FR261529

<table>
<thead>
<tr>
<th>IC_{50} (nM)</th>
<th>Human PARP Inhibition</th>
<th>Mouse PARP Inhibition</th>
<th>[3H]Spiroperone Binding</th>
<th>DAT Binding</th>
<th>TBARS</th>
<th>MAO</th>
<th>NOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR261529</td>
<td>33 ± 0.9</td>
<td>35 ± 1.1</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>3-AB</td>
<td>11,200 ± 810</td>
<td>11,800 ± 690</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Reference</td>
<td>Apomorphine</td>
<td>Nomifensine</td>
<td>Vitamin E</td>
<td>Pargyline</td>
<td>7-NID</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>223 ± 8.7</td>
<td>201 ± 6.8</td>
<td>228 ± 8.1</td>
<td>146 ± 5.1</td>
<td>319 ± 5.7</td>
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</table>

Results

PARP Inhibitory Activity of FR261529

To determine the species differences of PARP inhibitory activity of FR261529 and 3-AB, human recombinant PARP and nuclear extract from mouse brain were used. FR261529 potently inhibited the enzyme activity with an IC_{50} value of 33 ± 0.9 and 35 ± 1.1 nM in human and mouse PARP, respectively (Table 1). The inhibitory activity of FR261529 was about 400-fold more potent than that of 3-AB (IC_{50} values of 3-AB in this study were 11,200 ± 810 and 11,800 ± 690 nM in human and mouse PARP, respectively). To confirm the selectivity of FR261529 and 3-AB, PARP-2 enzyme assay
was also conducted. In our assay system, FR261529 was shown to be about 5-fold more potent for PARP-2 (IC\textsubscript{50} value of 7 nM) than PARP-1 (IC\textsubscript{50} value of 33 nM), compared with conventional nonselective PARP inhibitor 3-AB (IC\textsubscript{50} value for PARP-1 = 11.2 \mu M and for PARP-2 = 9.8 \mu M).

**Specificity of FR261529**

A number of drugs that affect DA metabolism, the dopamine receptor, and dopamine transporter or generation of ROS or NO can prevent METH neurotoxicity. Therefore, to exclude the possibility that FR261529 could inhibit METH neurotoxicity directly, MAO-B inhibitory activity, DAT binding affinity, and D2 receptor binding affinity were evaluated. In the MAO-B assay, FR261529 (IC\textsubscript{50} value of >10,000 nM) did not affect deamination of the substrate 2-\beta-ethyl-1-phenylethylamine in mouse brain homogenate, even with preincubation. Pargyline, which was used as a positive control, inhibited MAO-B activity with an IC\textsubscript{50} value of 146 nM. To determine the affinity of FR261529 to the dopamine transporter, a DAT binding assay was conducted using [\textsuperscript{3}H]mazindol as a radioligand. In this assay, FR261529 did not inhibit mazindol binding to DAT, even at a concentration of 10\textsuperscript{-5} M (IC\textsubscript{50} value of >10,000 nM), although nomifensine inhibited binding with an IC\textsubscript{50} value of 201 nM. Although apomorphine, a D2 agonist, inhibited spiperone binding with an IC\textsubscript{50} value of 201 nM, FR261529 showed no inhibitory activity even at 10\textsuperscript{-5} M (IC\textsubscript{50} value of >10,000 nM). Radical scavenging activity and NOS inhibitory activity were evaluated using a TBARS assay and NOS catalytic activity assay, respectively. In TBARS assay, FR261529 did not inhibit TBARS production up to 10\textsuperscript{-5} M (IC\textsubscript{50} value of >10,000 nM), although vitamin E showed radical scavenging activity with an IC\textsubscript{50} value of 228 nM. In the NOS assay, 7-nitroindazolazol, a selective nNOS inhibitor, prevented NOS catalytic activity assessed by [\textsuperscript{3}H]citrulline production with an IC\textsubscript{50} value of 319 nM, although FR261529 had no inhibitory activity up to 10\textsuperscript{-5} M (IC\textsubscript{50} value of >10,000 nM). 3-AB also showed very low affinity to DAT and D2 receptor, and no inhibitory activity to MAO and NOS. Radical scavenging activity of 3-AB was not observed in the TBARS assay (Table 1).

**Neuroprotective Action in PC12 Cells**

In this study, we first confirmed whether H\textsubscript{2}O\textsubscript{2} or METH treatment induced PARP activation and cell death in PC12 cells. PARP activation was confirmed by measurement of the amount of incorporation of [\textsuperscript{32}P]-NAD polymer in PC12 cells. Exposure of 0.1 to 1 mM H\textsubscript{2}O\textsubscript{2} for 30 min markedly increased incorporation of [\textsuperscript{32}P]-NAD polymer and treatment with FR261529 inhibited the PARP activation from a concentration of 10\textsuperscript{-6} M (Fig. 2A). METH treatment similarly induced formation of [\textsuperscript{32}P]-NAD polymer in PC12 cells (Fig. 2B). Treatment with FR261529 at concentrations higher than 10\textsuperscript{-6} M significantly inhibited METH-induced PARP activation. PARP activation by H\textsubscript{2}O\textsubscript{2} exposure was also observed when assessed by NAD assay, and excessive PARP activation resulted in marked NAD depletion, and this NAD depletion was significantly inhibited by FR261529 treatment (Fig. 2C).

Exposure of 0.1 mM H\textsubscript{2}O\textsubscript{2} for 6 h induced severe cell damage, although FR261529 treatment at a concentration range from 10\textsuperscript{-6} M significantly attenuated cell death as evaluated by MTT assay (Fig. 3A) and LDH assay (Fig. 3B). A similar neuroprotective effect was observed in PC12 cells treated with 3-AB at concentrations higher than 10\textsuperscript{-4} M (Fig. 3, C and D).

**Pharmacokinetic Study in Mice**

The plasma and brain concentrations of FR261529 were determined at 0.5 and 2 h after oral administration at a dose of 32 mg/kg. Mean plasma and brain concentration was 2.98
and 3.68 μg/g at 0.5 h and 0.83 and 1.49 μg/g at 2 h, respectively. Concentration of FR261529 in the brain was higher than that in the blood and the brain/plasma concentration ratio was 1.23 at Cmax time point (0.5 h after dosing) and 1.79 at 2 h after dosing.

Neuroprotective Action in Mouse METH Model

Neurotoxic Potential of METH. To determine a suitable dosing in the mouse METH model, the striatal content of DA was measured at 4 days after METH dosing. METH treatment (2×10, 2×15, 2×25, or 4×15 mg/kg, 2 h apart) caused significant and dose-dependent depletions in striatal DA by 30.6, 57.9, 79.7, and 90.6%, respectively, compared with the levels in saline-treated control (Table 2). These dosing paradigms also decreased DAT binding sites by 29.2, 57.4, 75.2, and 88.3%, respectively (Table 2). Changes in the striatal tissue levels of DA was coincident with the loss of striatal DAT binding sites and thus seems to represent the magnitude of dopaminergic terminal damage in the striatum.

DAT binding sites in the SNpc was also measured after METH dosing. Four doses of METH treatments dose dependently decreased DAT binding sites, and the degree of depletion was significant at higher doses (Table 2). These findings indicate that the dosing of METH (2×15 mg/kg, 2 h apart) produces moderate but significant dopaminergic neuronal damage and thus represents a suitable dosing paradigm to evaluate the potential of several compounds, including PARP inhibitors. In our preliminary studies, depletion of DA content in the striatum on day 4 after METH intoxication was comparable with that on day 14. Thus, the magnitude of dopaminergic terminals damage produced by METH could be stable even after 4 days. Therefore, the dosing paradigm and

**TABLE 2**

METH-induced dopaminergic neuronal damage in the striatum and the SNpc

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Assay</th>
<th>METH Dosing (i.p., 2-h interval)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>Striatum</td>
<td>DA content</td>
<td>100 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>DAT binding site</td>
<td>100 ± 4.5</td>
</tr>
<tr>
<td>SNpc</td>
<td>DAT binding site</td>
<td>100 ± 3.1</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01 vs. control by Dunnett’s test. *** P < 0.01, statistically significant compared with control group (by Student’s t test).
Brain PARP Inhibition by Treatment with FR261529. To explore the optimal dosing of FR261529 in mouse METH model, PARP inhibition by FR261529 treatment in mouse brain was determined by using PARP enzyme assay. FR261529 (3.2, 10, or 32 mg/kg) was orally administered in normal mice and nuclear extracts were prepared from mouse brain at 1 and 4 h after dosing. All doses of FR261529 significantly inhibited PAR polymer formation induced by addition of nicked DNA in the PARP enzyme assay at 1 h after dosing. Higher doses of FR261529 (10 and 32 mg/kg) moderately but still significantly inhibited PAR polymer formation at 4 h (Fig. 4).

Neuroprotective Effects of FR261529 and 3-AB. FR261529 was administered to mice at doses of 3.2, 10, or 32 mg/kg p.o. twice (1 h before first and 1 h after second injection of METH), and the striatum was dissected 4 days later to quantify DA and its metabolites. In this paradigm, treatment of FR261529 prevented the depletion of striatal DA, DOPAC, and HVA content in a dose-dependent manner, and with statistically significant protection at higher doses (10 and 32 mg/kg; Fig. 5A). 3-AB similarly significantly attenuated the depletion of DA and its metabolite contents at higher doses (320 mg/kg) (Fig. 5B). Furthermore, treatment of FR261529 ameliorated the damage of the nigrostriatal dopaminergic terminals in the striatum, as assessed by DAT binding (Fig. 5C). The depletion of DAT binding sites after METH intoxication and its percentage of recovery by FR261529 treatment was consistent with the degree of DA depletion and its recovery, respectively.

PARP Activation and Inhibition by FR261529 in the SNpc. PARP activation in the SNpc was under the detectable level at 2, 4, and 8 h after METH intoxication (2 × 15 mg/kg, 2 h apart), assessed by PAR polymer formation using nuclear extracts from mouse brain (data not shown). However, more severe dosing of METH (4 × 15 mg/kg, 1 h apart) produced significant PARP activation in the SNpc 2 h after the last injection of METH. This PARP activation was significantly prevented by FR261529 treatment (2 × 32 mg/kg, 5 h apart) as shown in Fig. 6.

METH-Induced Lipid Peroxidation. To determine the level of lipid peroxidation after METH intoxication, the amount of MDA, a lipid marker of oxidative stress, was measured in the striatum, hippocampus, and cortex using TBARS assay. METH treatment (2 × 15 mg/kg, 2 h apart) resulted in regionally selective and time-dependent elevation of MDA. Elevation of MDA was detectable in the striatum at 2 h and persisted at 24 h after METH treatment (Fig. 7A), but it was not observed in the hippocampus and cortex at both time points. Administration of DAT inhibitor nomifensine (3.2 mg/kg) and the DA antagonist haloperidol (3.2 mg/kg) significantly antagonized the elevation of MDA at 2 h after METH injections. On the other hand, the PARP inhibitors FR261529 and 3-AB did not prevent the elevation of MDA induced by METH treatment at neuroprotective doses (Fig. 7B).

Effect on DA Metabolism in the Striatum. The efficacy of several drugs with protective effects against METH toxicity was analyzed by measuring DA and its metabolite contents 1 h after METH injection. Consistent with previous reports (Cosi et al., 1996; Fornai et al., 2001), administration of a DAT inhibitor (nomifensine, 3.2 mg/kg i.p.), a D2 receptor agonist (apomorphine, 3.2 mg/kg i.p.), or a D2 receptor antagonist (haloperidol, 3.2 mg/kg i.p.) affected DA and its metabolite contents at 1 h after dosing (Table 3). In contrast, FR261529 administration did not modify striatal DA, DOPAC, or HVA levels (Table 4).

Effects on Body Temperature. Rectal temperature was significantly elevated within 1 h after the first injection of METH (2 × 15 mg/kg, 2 h apart) compared with the saline-treated control mice. This hyperthermia by METH was maintained until 3 h after the second injection of METH, and all doses of FR261529 (3.2, 10, and 32 mg/kg; twice, 4 h apart) did not induce a significant hypothermic effect (Fig. 8).

Discussion

A novel PARP inhibitor FR261529, which was designed through a structure-based drug design system using the crystal structure of human recombinant PARP, showed potent inhibitory activity of both human recombinant PARP and PARP in murine nuclear extracts. Other newly synthesized quinazoline derivatives also show no significant species difference between human PARP and mouse PARP inhibitory activity (data not shown), consistent with our previous data that quinazolino derivatives showed no species differences on its inhibitory activity (Iwashita et al., 2004a,b), as well as with reports that the PARP catalytic domain shows the highest degree of homology between different species (de Murcia et al., 1994). Furthermore, more interestingly, FR261529 was more potent for PARP-2 than PARP-1 (5-fold higher selectivity) compared with nonselective general PARP inhibitors such as 3-AB, indicating that FR261529 and its derivatives could be the first class of PARP inhibitors that have some degree of selectivity for PARP-2 among several published PARP inhibitors (unpublished data). However, further
detailed studies would be required to address pharmacological potentials of PARP-1 and/or PARP-2 inhibition.

METH enters DA terminals via the DAT and displaces both vesicular and intracellular DA. This results in increased DA release within the cytoplasm of DA terminals, where DA is oxidized by MAO activity and auto-oxidation to ROS and hydrogen peroxide (Cubells et al., 1994; Cadet and Brannock, 1998). METH administration can also lead to increased extracellular glutamate (Nash and Yamamoto, 1992), which, in turn, induces NMDA receptor activation, linking to the formation of superoxide and nitric oxide (Lafon-Cazal et al., 1993; Gunasekar et al., 1995; Yamamoto and Zhu, 1998). Superoxide may react with nitric oxide produced by NOS to form the potent oxidant peroxynitrite, which damages intra-
FR261529 prevented PARP activation completely. PARP activations rep-
dosings of METH significantly increased PARP activation, and treatment
and determined PARP activation used by PARP enzyme assay. Four
was dissected from each mouse at 1 h after first injection and 1 h after fourth injection of METH. The SNpc
1 h before first injection and 1 h after fourth injection of METH. The SNpc
intervals and FR261529 (32 mg/kg p.o.) was administered twice by p.o. at
SNpc. METH (15 mg/kg) was intraperitoneally injected four times at 1-h
properties or nNOS inhibitory activity, even at a concentration
overexpressing the antioxidant enzyme copper/zinc superoxide
dismutase showed an attenuated response to neurotoxic doses
mice lack the nNOS gene are resistant to
METH-induced dopaminergic neurotoxic-
ity, neuroprotection by various nNOS inhibitors, along with the
evidence that mice lacking the nNOS gene are resistant to
METH-induced dopaminergic neurotoxicity have been reported
(Di Monte et al., 1996; Itzhak et al., 1998). Furthermore, the
administration of antioxidants can attenuate METH-induced
neurotoxicity (De Vito and Wagner, 1989), and transgenic mice
overexpressing the antioxidant enzyme copper/zinc superoxide
dismutase showed an attenuated response to neurotoxic doses of
METH (Cadet et al., 1994). These results indicate that radical
scavengers and nNOS inhibitors can block METH-induced
dopaminergic neuron damage. FR261529 had no antioxidant
properties or nNOS inhibitory activity, even at a concentration
10^{-5} M, suggesting that neither radical scavenging activity nor
nNOS inhibition is involved in the action of FR261529.
Although the effects of FR261529 on other diverse classes of
known neurotransmitter receptors, ion channels, or enzymes
have not been fully clarified, the neuroprotective properties are
likely the consequence of its specific PARP inhibitory activity.
To determine PARP inhibitory and neuroprotective properties
of FR261529 in cultured cells, PARP activation and cell
damage were induced by H$_2$O$_2$ or METH exposure in PC12
cells. In this cell death system, H$_2$O$_2$ exposure markedly
induced PARP activation, concomitant NAD depletion, and
early stage cell death. Inconsistent with the potent PARP
inhibitory activity of FR261529 (IC$_{50}$ = 33 nM), this com-
 pound attenuated both PARP activation and cell death at a
concentration higher than 1000 nM (30-fold difference), sug-
gesting that FR261529 does not have good cell permeability
compared with that of 3-AB, another widely used PARP
inhibitor (IC$_{50}$ = 11.2 μM versus minimum effective concentra-
tion = 100 μM; <10-fold difference) and quinoxaline
derivatives (<10-fold difference; data not shown). Other quino-
xaline derivatives of FR261529 also showed a discrepancy
between PARP inhibitory activity in enzyme assay and
neuroprotective properties in cultured cells (data not shown).
Therefore, we speculate that the relatively low potency of
neuroprotective properties of FR261529 and its derivatives in
mouse METH model results from, in part, its poor perme-
ability across the cell membrane.

METH-induced dopaminergic neuronal damage, assessed
by DA and metabolite contents, was well correlated with the
reduction of DAT binding sites in the striatum. Although the
reduction of DAT binding sites in the SNPc was relatively

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reduction of DAT binding sites in the striatum. Although the
reduction of DAT binding sites in the SNPc was relatively

![Fig. 6. Effects of FR261529 on METH-induced PARP activation in the
SNPc. METH (15 mg/kg) was intraperitoneally injected four times at 1-h
intervals and FR261529 (32 mg/kg p.o.) was administered twice by p.o. at
1 h before first injection and 1 h after fourth injection of METH. The SNPc
was dissected from each mouse at 1 h of last administration of FR261529
determined PARP activation used by PARP enzyme assay. Four
dosings of METH significantly increased PARP activation, and treatment
FR261529 prevented PARP activation completely. PARP activations rep-
resent the incorporation of $^{32}$P-NAD polymer (297 ± 20.8 cpm/10 mg of
brain tissue in the nontreated control group). Data are presented as
mean ± S.E.M of five to six mice. Significant difference from control value
are indicated by *, P < 0.05 versus vehicle-treated control group and by
#, P < 0.05 versus FR261529-treated group (by Student’s t test).

![Fig. 7. METH-induced lipid peroxidation in the striatum. The degree of
lipid peroxidation following METH treatment was measured using by
TBARS assay. A, METH treatment (2 × 15 mg/kg, 2 h apart) time
dependently increased the generation of MDA in the striatum specifi-
cally, and its effect persisted on until 24 h after last METH treatment. B,
administration of nomifensine or haloperidol, which has protective po-
tential in mouse METH model, significantly attenuated the MDA pro-
duction at 2 h after METH treatment. However, neuroprotective dose of
3-AB or FR261529 did not show any change of MDA production. Data are
presented as mean ± S.E.M of five to six mice. Significant difference from
control value is indicated by **, P < 0.01, statistically significant com-
pared with control group (by Student’s t test).]
Dopamine and its metabolites contents at 1 h following drug administration

**TABLE 4**

<table>
<thead>
<tr>
<th>Drug Route</th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
<th>3-MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>METH</td>
<td>106.1 ± 5.6</td>
<td>42.8 ± 1.4**</td>
<td>156.5 ± 9.1**</td>
<td>361.5 ± 21.6**</td>
</tr>
<tr>
<td>FR261529</td>
<td>96.1 ± 3.9</td>
<td>95.8 ± 7.8</td>
<td>104.3 ± 3.8</td>
<td>98.8 ± 3.2</td>
</tr>
<tr>
<td>Nomifensine</td>
<td>95.8 ± 4.2</td>
<td>54.2 ± 2.2**</td>
<td>77.5 ± 2.2**</td>
<td>94.2 ± 6.6</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>50.9 ± 2.1**</td>
<td>386.3 ± 18.1**</td>
<td>209.5 ± 9.4**</td>
<td>53.6 ± 5.1**</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>98.7 ± 1.8</td>
<td>62.9 ± 5.4**</td>
<td>49.2 ± 4.6**</td>
<td>67.8 ± 3.1**</td>
</tr>
</tbody>
</table>

**Fig. 8.** Effect of FR261529 on body temperature during METH treatment in mice. METH-induced hyperthermia was observed immediately after first injection of METH treatment (2 × 15 mg/kg, 2 h apart) and persisted on 4 h later. Treatment of FR261529 at doses from 3.2 to 32 mg/kg did not show hyperthermic action and did not affect hyperthermia of METH treatment. Data are presented as mean ± S.E.M. of 5 to 6 mice.
ence on MDA generation after METH treatment. These results offer supportive evidence that METH produces oxidative damage in vivo but that FR261529 does not inhibit the generation of METH-induced radical formation.

Body temperature has also been found to markedly influence METH-induced DA neurotoxicity. METH-induced formation of ROS is temperature-sensitive and is attenuated by hypothermia (Bowyer et al., 1992; Ali et al., 1994). These findings suggest that hyperthermia facilitates formation of oxidative species resulting from the administration of high-dose METH treatment, whereas hypothermia is neuroprotective. Severe hyperthermia may result in the activation of or up-regulation of proteins capable of oxidizing DA and formation of DA quinine (LaVoie and Hastings, 1999). In this study, FR261529 did not affect METH-induced hyperthermia or METH-induced lipid peroxidation in the striatum, providing evidence that neuroprotective effect of FR261529 is not due to inhibition of SOD and/or NO production directly.

In conclusion, a newly synthesized PARP inhibitor, FR261529, exhibited potent PARP inhibition both in vitro and in vivo, with significant neuroprotective activity after METH intoxication in mice, suggesting that nigro-striatal dopaminergic neurodegeneration induced by METH involves PARP activation and therefore this METH model might be a useful neurodegenerative model in experimental animals to evaluate neuroprotective properties of PARP inhibitors. Furthermore, the orally active and brain penetrable PARP inhibitor FR261529, or one of its derivatives, could be not only an important tool for investigating the physiological role of PARP in neurodegenerative pathways but also an attractive therapeutic candidate for neurodegenerative disorders such as Parkinson’s disease.

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References


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