Neuroprotective Effects of a Novel Poly(ADP-Ribose) Polymerase-1 Inhibitor, 2-{3-[4-(4-Chlorophenyl)-1-piperazinyl] propyl}-4(3H)-quinazolinone (FR255595), in an in Vitro Model of Cell Death and in Mouse 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine Model of Parkinson’s Disease

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ABSTRACT

The massive activation of poly(ADP-ribose) polymerase-1 (PARP-1) by DNA-damaging stimuli, such as exposure to reactive oxygen species (ROS), can lead to cell injury via severe, irreversible depletion of the NAD and ATP pool, and PARP-1 inhibitors have been expected to rescue neurons from degeneration in a number of disease models. We have recently identified 2-{3-[4-(4-chlorophenyl)-1-piperazinyl] propyl}-4(3H)-quinazolinone (FR255595) as a novel and potent PARP-1 inhibitor through structure-based drug design and high-throughput screening. This compound potently inhibited PARP activity with an IC50 value of 11 nM and was orally active and highly brain penetrable. Here, we show that prevention of PARP activation by FR255595 protects against both ROS-induced cell injury in vitro and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal dopaminergic damage in an in vivo Parkinson’s disease (PD) model. In cell death models in vitro, exposure of hydrogen peroxide induced cell death with PARP overactivation in PC12 cells and SH-SY5Y cells, and pre- and post-treatment with FR255595 (10–100–1000 M) significantly reduced PARP activation and cell death. In mouse MPTP model, MPTP (20 mg/kg i.p.) intoxication lead to PARP activation and cell damage in the nigrostriatal dopaminergic pathway, which was significantly ameliorated by oral administration of FR255595 (10–32 mg/kg), both in the substantia nigra and in the striatum via marked reduction of PARP activation, even with delayed treatment. These findings clearly indicate that the novel PARP-1 inhibitor FR255595 exerts neuroprotective effect through its potent PARP-1 inhibitory actions in PD model, suggesting that the drug could be an attractive candidate for several neurodegenerative disorders, including PD.

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme and normally functions in DNA repair, but extensive PARP activation can promote cell death through processes involving energy depletion. It has been reported that reactive oxygen species (ROS)-mediated damage of DNA can activate PARP (Berger, 1985; Szabo and Dawson, 1998) and consumes NAD and consequently ATP, culminating in cell dysfunction or necrosis (Ha and Snyder, 1999). On the other hand, PARP plays a central role in a caspase-independent apoptosis pathway mediated by apoptosis-inducing factor (Yu et al., 2002). Translocation of apoptosis-inducing factor from the mitochondria to the nucleus is dependent on PARP activation in neurons treated with various DNA-damaging stimuli such as N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-D-aspartate, 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 such as Parkinson’s disease (PD), a chronic progressive neurologic

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; PD, Parkinson’s disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FR255595, 2-{3-[4-(4-chlorophenyl)-1-piperazinyl] propyl}-4(3H)-quinazolinone; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; MAO-B, monoamine oxidase-B; DAT, dopamine transporter; KRB, Krebs-Ringer-HEPES; TBARS, thiobarbituric acid reactive substances; NOS, nitric-oxide synthase; SNpc, substantia nigra pars compacta; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; nNOS, neuronal nitric-oxide synthase; TH, tyrosine hydroxylase.
disorder related to the degeneration of the neurons in the substantia nigra, and PARP inhibitors have been shown to be effective in animal models of stroke, traumatic brain injury, and Parkinson’s disease (Cosi et al., 1996; Takahashi et al., 1999; Abdelkarim et al., 2001; LaPlaca et al., 2001).

The synthetic heroin analog, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can selectively damage neurons in the nigrostriatal dopaminergic pathway and produce parkinsonism in humans and in experimental animals (Kopin and Markey, 1988; Langston, 1996; Przedborski and Jackson-Lewis, 1998; Blum et al., 2001). There is evidence for both the production of reactive oxygen intermediates (Przedborski et al., 1996; Cassarino et al., 1997; Hung and Lee, 1998) and nitric oxide-derived radicals/oxidants in the pathogenesis of MPTP neurotoxicity. Direct evidence for the involvement of PARP in the pathogenesis of neurotoxicity induced by MPTP comes from a mouse model of PD (Mandir et al., 1999). MPTP intoxication reduces striatal dopamine and cortical noradrenal in levels by more than 50% in C57BL/6 mice, whereas treatment of conventional PARP-1 inhibitors ameliorates the depletion induced by MPTP (Cosi et al., 1996). The protective potency of the PARP inhibitor benzamide and its derivatives parallels their inhibitory activities on PARP enzyme activity (Cosi et al., 1996). Furthermore, recent studies have demonstrated that dopaminergic neurons from mutant mice lacking the gene coding PARP are also partially but dramatically spared from the neurotoxic effect of MPTP (Mandir et al., 1999). MPTP exposure induces many of the biological and neuropathological changes in the nigrostriatal dopaminergic pathway that are observed in post-mortem studies of PD patients. This evidence suggests that MPTP intoxication induces nigrostriatal dopaminergic neurodegeneration and parkinsonism in mice via PARP activation partly as a result of ROS generation and that PARP inhibition will result in amelioration of MPTP-induced neurotoxicity. Therefore, PARP inhibitors could possess a therapeutic potential for the treatment of neurodegenerative disorders and several other diseases involved in PARP activation.

We have recently identified FR255595 as a novel and potent PARP-1 inhibitor. The purpose of the present study was, first, to investigate the PARP-1-inhibiting activity and specificity of FR255595. The second purpose of the present study was to determine the PARP-1 inhibitory properties and the neuroprotective properties of FR255595 in vitro experimental neuronal cell death models, in which PARP-1 is markedly activated by H2O2 exposure. Finally, the neuroprotective properties of FR255595 were evaluated in a mouse MPTP model, which is a pertinent in vivo model of PD.

Materials and Methods

Materials

Rat pheochromocytoma PC12 cells and human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA). 2-[3-[4-(4-Chlorophenyl)-1-piperazinyl] propyl]-4(3H)-quinazolinone (FR255595; chemical structure shown in Fig. 1) was synthesized at Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). MPTP, tissue culture medium, and fetal bovine serum were purchased from Sigma-Aldrich. Inc. (Gaithersburg, MD), and recombinant mouse PARP-2 enzyme was purchased from Alexis Biochemicals (San Diego, CA). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich.

Measurement of PARP Inhibitory Activity in Vitro

To assess the PARP-1 or PARP-2 inhibitory activity of FR255595, 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 MgCl2, 1 mM dithiothreitol, 10 μg of activated salmon sperm DNA, 0.1 μCi of [adenylate-32P]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 FR255595. 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 Cultured Cells and the Rat/Mouse Brain

For preparation of nuclear extracts, published methods were used as described previously with minor modifications (Lahiri and Ge, 2000). To prepare the nuclear extracts from the cultured cells, 2 million cells cultured in F25 flask were washed with 10 ml of phosphate-buffered saline (PBS), and cells were resuspended in 500 μl of cold buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, and 0.1% Nonidet-P40) and homogenized gently. The homogenate was centrifuged at 5000g 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 on a micro tube mixer for 15 min at 4°C. 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-1 assay immediately.

For preparation of nuclear extracts from rat and mouse brain, normal and/or drug-treated whole brains were dissected and transferred to Teflon homogenizer. The 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 the Eppendorf tubes followed by centrifugation (1600g) in a microcentrifuge at 4°C 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 micro tube 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-1 enzyme and DNA was used for PARP-1 assay immediately.

Specificity of FF255595

Determination of MAO-B Inhibitory Activity. MAO-B activity in mouse brain was determined by radioassay as described by...
Wurtman and Axelrod (1963) with minor modifications using [14C]-2-
β-ethyl-1-phenylethylamine HCl (about 50 mCi/mmol; PerkinElmer Life
Sciences, Boston, MA) as substrates at concentrations of 20.8
µM (specific activity 1.6 mCi/µmol). Briefly, mitochondrial homoge-
nate from mouse brain in 85 µl of 67 mM sodium phosphate buffer
(pH 7.4) and 5 µl of desired concentrations (10⁻⁸-10⁻⁵ M) of the
compounds were preincubated in 96-well plates at 37°C for 10 min.
After the preincubation, 10 µl of [14C]-β-ethyl-1-phenylethylamine
was added and incubated at 37°C for 20 min under gentle shaking.
The reaction was terminated by placing the plate on wet ice and
adding 100 µl of cold 3 M HCl. The deaminated products were
extracted by 0.5 ml of ethyl acetate, and the separation of the two
phases was facilitated by centrifugation (700g) at 15°C for 10 min.
The samples were analyzed in the scintillation counter for the
amount of 14C in the upper phase.

**Dopamine Transporter (DAT) Binding Assay.** To determine the
affinity of FR255595 to DAT, and also to evaluate the level of cell
damage after MPTP intoxication in mice, the striatum and/or sub-
stantia nigra were dissected from mouse brains, and homogenate
with KRH buffer containing 125 mM NaCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.6 mM glucose, 0.01 mM
nialamide, and 25 mM HEPES, pH 7.4, was prepared by several
centrifugations. For DAT binding assay, mazindol binding to the
DAT was evaluated according to the method of Javitch et al. (1985)
with minor modifications. Binding was assayed by addition of mem-
branes preparation from the striata or substantia nigra, about 125
µg of protein, to an incubation solution (KRH buffer) containing 5 nM
[^3H]mazindol (17 Ci/µmol; PerkinElmer Life Sciences) and 300
µM 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 bind-
ing was determined in the presence of 10 µM benztropine or cocaine.

**Determination of Radical Scavenging Activity.** For measure-
ment of lipid peroxidation, thiobarbituric acid reactive substances
(TBARS) were estimated using the modified method of Buege and
Aust (1978) and Callaway et al. (1998). Briefly, mouse brain synap-
somes were prepared from C57BL/6 mice (Japan SLC, Shizuoka,
Japan). To evaluate the inhibitory activity of FR255595, different
concentrations of the compounds was dissolved 50% dimethyl sulfox-
ide, and then 5 µl was added to each rat brain synaptosome and
incubated with ammonium ferric sulfate (100 µM) at 37°C for 30
min. The reaction was stopped with addition of 20% TCA, and the
precipitated proteins were removed by centrifugation at 10,000g for
15 min. The aliquots of supernatant were then added to an equal
volume of thioarbituric acid. 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. Percentage of inhibition of TBARS
production was calculated as follows: percentage of inhibition =
((Max – Drug)/(Max – Base)) x 100, where Max is the value in the
presence of ammonium ferric sulfate, Base is the value in the ab-
sence of ammonium ferric sulfate, and Drug is the values of test
compounds.

**Determination of NOS Inhibitory Activity.** NOS catalytic ac-
tivity was assayed by measuring the Ca²⁺-dependent conversion of
[^3H]arginine to[^3H]citrulline as described by Huang et al. (1998). For
this assay, dissected mouse brain was homogenized in 20 volumes
(w/v) of 25 mM Tris buffer (pH 7.4) containing 1 mM EDTA and 1
mM EGTA. After centrifugation (20,000g for 15 min at 4°C), 25 µl of
supernatant was added to 75 µl of 50 mM Tris buffer (pH 7.4)
containing 1 mM NADPH, 1 mM EDTA, 3 mM CaCl₂, and 0.1 µCi of
[^3H]arginine (specific activity 64 Ci/µmol; PerkinElmer Life Sci-
ences) in the absence or presence of FR255595 solution and incu-
bated for 15 min at 37°C. The reaction was terminated by the addition
of 250 µl of Dowex AG50WX-8 (Pharmacia, Peapack, NJ) and
cooled on ice. After centrifugation,[^3H]citrulline was quantified
by liquid scintillation counting of 100 µl of supernatant. No signifi-
cant[^3H]citrulline production occurred in the absence of calcium.

**Neuroprotective Efficacy in PC12 Cells and SH-SY5Y Cells.**

**Cells and Hydrogen Peroxide Treatment.** PC12 cells and SH-
SY5Y 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 antibiotics mixture. Cells were grown in an atmosphere
of 95% air and 5% CO₂ at 37°C for 24 h. For all experiment, cells were
seeded at a density of 4 × 10⁵ cells/well in 96-well culture plates and
allowed to attach overnight. The cells were incubated with or without
hydrogen peroxide (100 or 300 µM) and FR255595 for 6 h. To evaluate
cell survival, a MTT assay was performed according to a minor modi-
fication of the original method (Mosmann, 1983). 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 0.4 N HCl.
The absorbance of each well was measured at 590 nm by using a
microplate reader (Molecular Devices, Sunnyvale, CA).

**Drug Treatment.** FR255595 was dissolved in 100% dimethyl
sulfoxide at 10⁻³ M and then diluted in Dulbecco’s modified Eagle’s
medium without serum. This solution was added to culture plate
0.5 h before H₂O₂ exposure.

**Determination of Cellular NAD Level.** To determine NAD
level in cultured cells, PC12 cells were seeded at 2 × 10⁵ cells/well in
24-well plates and cultured for 24 h. FR255595 was added to cell
media at several concentrations. Thirty minutes later, cells were
exposed 100 µM H₂O₂ for 30 min, and cells were detached using
by cell scraper and then collected in microcentrifuge tube by cen-
trifugation for 5 min × 100g at 4°C. To quantify the NAD level in mouse
brain, brain homogenate (10 mg of tissue/150 µl in PBS) dissected
from the striatum and the substantia nigra was prepared, respec-
tively. Cells or brain 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.

**Determination of PARP Activation.** To determine the PARP
activation directly in PC12 cells, PARP-1 enzyme assay was used.
PC12 cells were seeded at 2 × 10⁵ cells in F75 culture flask and
cultured for 24 h. Then, FR255595 was added to culture media at several
concentrations. Thirty minutes later, cells were exposed 100
µM H₂O₂ for 30 min, and cells were detached using cell scraper and
then nuclear extracts were prepared as described above. PARP acti-
vation was determined by PARP-1 enzyme assay using nuclear ex-
tracts as a mixture of PARP-1 enzyme and nicked DNA.

**Poly(ADP-Ribose) Polymer Western Blots.** Western blot anal-
ysis of poly(ADP-ribose) polymer was performed using PC12 cells
lysates. Samples containing 20 µg of protein were loaded, separated
on a 5 to 20% of polyacrylamide gel, and transferred to a nitrocellu-
lose membrane. The membrane was incubated with anti-poly(ADP-
ribose) polyclonal antibody and stained with Ponceau S (0.1%) to
confirm equal loading and transfer. After blocking of nonspecific
sites by a 10% of nonfat dry milk in PBS for 30 min, membranes were
incubated with rabbit anti-poly(ADP-ribose) polyclonal antibody.
Bands were visualized by chemiluminescence.

**MPTP-Induced Parkinson’s Model in Mice.**

**Animals.** For the MPTP model, 9- to 10-week-old male C57BL/6
mice, weighing 19 to 22 g (Charles River, Hino, Japan), 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 weeks before the
experiment. Animals were housed five per cage and allowed free
access to food and water. All experiments in the present study were
performed under the guidelines of the Experimental Laboratory
Animal Committee of Fujisawa Pharmaceutical Co., Ltd. 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 both the number of animals used and stress to the animals during experimental procedures.

Administration of MPTP and FR255595. To determine the neuroprotective properties of FR255595 in in vivo mouse MPTP model, two experimental paradigms of MPTP delivery were used as described by Jackson-Lewis et al. (1995) with minor modifications. One is the four-dose paradigm of MPTP intoxication to induce severe cell injury, and the other is the two-dose paradigm to induce milder cell injury. The animals received 4 (severe model) or 2 (mild model) injections of FR255595 in normal C57BL/6 mice at 3.2 to 32 mg/kg p.o. once, and each brain was dissected at different time points (2, 8, and 24 h) after a single p.o. administration of FR255595. The brain homogenates were prepared immediately, and PARP-1 assay was conducted as described above.

Preparation of Substantia Nigra and Striatum Homogenate. For NAD assay and DAT binding assay for tissue homogenates, whole brains were dissected and put on ice-cold brain slicer immediately. A 1-mm-thick slice including SNpc and whole bilateral striatal area was sectioned precisely by referring to a mouse brain map. The exact SNpc area was also confirmed by comparison with the result of TH immunostaining, and the TH-negative area was removed from the slice precisely. The remaining tissues were used for preparation of the homogenate.

Measurement of PARP-1 Inhibition in Mouse Brain. To evaluate the inhibition of PARP-1 activity in the brain after the treatment of FR255595, FR255595 was administered in normal C57BL/6 mice at 3.2 to 32 mg/kg p.o. once, and each brain was dissected at different time points (2, 8, and 24 h) after a single p.o. administration of FR255595. The brain homogenates were prepared immediately, and PARP-1 assay was conducted as described above.

Measurement of Striatal DA, DOPAC, and HVA Levels. The contents of DA, DOPAC, and HVA in the striatum were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. Four days after 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 M EDTA-2Na containing 50 ng/ml isoproterenol as internal standard. After centrifugation (15,000g, 15 min, 4°C), pH of supernatant from each sample was adjusted to 3.5 with sodium acetate. Adjusted supernatants were filtered and injected onto a reverse phase catecholamine column (SL-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. Dopamine and its metabolites were quantified by peak height comparisons with standards run on the day of analysis.

Pharmacokinetic Study in Mice

Measurement of the concentration of FR255595 in plasma and brain were performed in C57BL/6 mice after p.o. administration at 32 mg/kg. FR255595 was suspended in 0.5% methylcellulose and administered orally in a volume of 10 ml/kg. The plasma and brain were collected at 0.5 h and 2 h after dosing, and the plasma and brain levels of FR255595 were measured using HPLC. For measurement of MPP⁺ levels in the brain, FR255595 (32 mg/kg) was administered orally, and 1 h after dosing, MPTP (20 mg/kg i.p.) was administered. The brain was collected at 0.5 h and 2 h after dosing, and MPP⁺ level in the brain was measured by HPLC. A 200-μl plasma sample was extracted with 400 μl of acetonitrile, and the organic phase was separated. Whole brain was homogenized in 1 ml of saline, and the 200-μl sample was further homogenized in 400 μl of acetonitrile solution. Each supernatant was subsequently evaporated under a gentle stream of nitrogen, and the dried residue was reconstituted in 150 μl of 50% acetonitrile. The 35-μl volume of reconstitute was injected onto a reverse phase column (Inertsil ODS-3, 4.5 × 150 mm, i.d., 5 μm; GL Science, Tokyo, Japan) and detected using a Waters model 486 tunable absorbance detector in UV mode at λ max of 225 nm. The mobile phase consisted of 40% (v/v) acetonitrile in 20 mM KH2PO4 buffer.

Statistical Analysis

The IC50 values obtained from studies in vitro were calculated using GraphPad Prism 3.3 software (GraphPad Software Inc., San Diego, CA). All values are expressed as mean ± S.E.M. Statistical significance of difference between groups was tested using one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s multiple comparison test. P values less than 0.05 were considered to be significant.

Results

PARP-1 and PARP-2 Inhibitory Activity of FR255595

For PARP-1 enzyme assay, the nuclear extracts mainly containing PARP-1 enzyme and DNA were prepared from rat and mouse brain. The existence of PARP-1 enzyme and DNA in the nuclear extracts was confirmed by Western blot analysis and increase of PARP activity after addition of DNase I in the PARP-1 enzyme assay, respectively (data not shown). To compare the species differences of PARP-1 inhibitory activity of FR255595, human recombinant PARP-1 and nuclear extracts from rat and mouse brain were used as rat PARP-1 and mouse PARP-1. FR255595 was able to potently inhibit the enzyme activity with an IC50 of 11 ± 0.4, 13 ± 0.6, and 12 ± 0.5 mM in human, rat, and mouse PARP, respectively (Fig. 2A). Furthermore, FR255595 was shown to be more selective to PARP-1 (IC50 = 11 mM) than PARP-2 (IC50 = 300 mM) compared with nonselective general PARP inhibitor such as 3-aminobenzamide (IC50 = 10 μM for PARP-1 versus IC50 = 9 μM for PARP-2).

Specificity of FR255595

A number of drugs that affect MPTP metabolism can prevent MPTP neurotoxicity. Therefore, to determine whether FR255595 has properties to inhibit MPTP neurotoxicity directly, MAO-B inhibitory activity and DAT binding affinity were evaluated. In the MAO-B assay, FR255595 (10⁻⁸–10⁻⁵ M) did not affect deamination of the MAO-B substrate [³⁵C]2-β-ethyl-1-phenylethylamine HCl in rat brain homogenate, even with preincubation (Fig. 2B). Pargyline, which was used as a positive control, inhibited MAO-B activity from the concentrations of 10⁻⁷ M (Fig. 2B). To determine the affinity of FR255595 to the dopamine transporter, DAT binding assay was conducted using [³⁵H]mazindol as a radioligand. In this assay, FR255595 did not inhibit mazindol binding to DAT even at a concentration of 10⁻⁵ M, whereas nomifensine, which was used as positive control, inhibited at concentrations higher than 10⁻⁷ M (Fig. 2C). Furthermore, radical scavenging activity and NOS inhibitory activity was evaluated using TBARS assay and NOS catalytic activity assay, respectively. In TBARS assay, FR255595 did not inhibit TBARS production, even at a concentration of 10⁻⁵ M, although vitamin E showed radical scavenging activity at 10⁻⁵ M (Fig. 2D). In NOS assay, 7-nitroindazole, a selective nNOS inhibitor, prevented NOS catalytic activity assessed by [³⁵H]citrulline production in the concentration ranging from
Neuroprotective Action in PC12 Cells and SH-SY5Y Cells

In this study, we first confirmed whether H₂O₂ treatment induced PARP activation, as well as concomitant NAD depletion and cell death in PC12 cells and SH-SY5Y cells. PARP activation was confirmed by measurement of the amount of incorporation of [³²P]NAD polymer and by detection of poly- (ADP-ribose) polymer (PAR polymer) formation using Western blot analysis in PC12 cells. H₂O₂ (100 μM) exposure for 30 min markedly increased incorporation of [³²P]NAD poly-
mer (Fig. 3A) and induced massive formation of PAR polymer in PC12 cells (Fig. 3B). Treatment with FR255595 at a concentration of $10^{-5}$ M completely inhibited incorporation of [32P]NAD polymer and PAR polymer formation as shown in Fig. 3A and in lane 3 of Fig. 3B, respectively. PARP activation by H$_2$O$_2$ exposure was also confirmed in NAD assay. Excessive PARP activation resulted in marked NAD depletion (Fig. 3C), and this NAD depletion was completely inhibited by FR255595 treatment ($10^{-9}$–$10^{-5}$ M). Exposure of H$_2$O$_2$ for 6 h induced severe cell damage, although FR255595 treatment at a concentration range from $10^{-9}$ to $10^{-5}$ M significantly attenuated cell death (Fig. 3D).

In SH-SY5Y cells, H$_2$O$_2$-induced PARP activation and NAD depletion were also observed (data not shown), and

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Fig. 3. Hydrogen peroxide-induced PARP activation, NAD depletion, and cell death was prevented by PARP inhibition with FR255595 treatment in PC12 cells. Exposure of 100 µM H$_2$O$_2$ for 30 min induced marked PARP activation, evaluated by the incorporation of [32P]NAD polymer, and treatment with FR255595 (10 µM) completely inhibited PARP activation (A). PARP activation was also detected by using Western blot after H$_2$O$_2$ exposure (100 µM) for 30 min in PC12 cells. PAR polymer formation in the vehicle-treated group was under detectable level (lane 1); however, H$_2$O$_2$ exposure induced marked PAR polymer formation represented as 116-kDa protein (lane 2). This PAR polymer formation was completely prevented by addition of 10 µM FR255595 (lane 3). This photograph is representative of three analyses (B). FR255595 at concentrations from $10^{-9}$ to $10^{-5}$ M attenuated NAD depletion, and at a $10^{-5}$ M concentration, FR255595 prevented NAD depletion completely (C). Exposure of 100 µM H$_2$O$_2$ for 6 h produced severe cell damage, evaluated by MTT assay. This damage was significantly reduced by addition of $10^{-5}$ to $10^{-5}$ M FR255595 0.5 h before H$_2$O$_2$ exposure to the culture medium (D). Each point represents the mean ± S.E.M. of at least three experiments. **, $P < 0.01$ versus vehicle-treated control group (by one-way ANOVA followed by Dunnett’s multiple comparison test). ##, $P < 0.01$ versus vehicle-treated control group (by Student’s t test).
treatment with FR255595 (10⁻⁹–10⁻⁶ M) 30 min before exposure of H₂O₂ significantly prevented the NAD reduction and neuronal cell death (Fig. 4A). This neuroprotective effect was even observed when FR255595 was added after the onset of PARP activation (2 h after H₂O₂ exposure; Fig. 4B).

**Pharmacokinetic Study in Mice**

The plasma and brain concentrations of FR255595 were determined at 0.5 h and 2 h after oral administration at a dose of 32 mg/kg in C57BL/6 mice. Mean plasma and brain concentrations was 2.37 and 6.54 μg/g at 0.5 h, and 1.69 and 2.73 μg/g at 2 h, respectively. Concentration of FR255595 in the brain was significantly higher than that in the blood, and the brain/plasma concentration ratio was 2.76 at Cmax time point (0.5 h after dosing).

**MPP⁺ Levels in the Brain**

To confirm that the neuroprotective effect of FR255595 is not caused by reduced metabolism of MPTP to MPP⁺, we measured MPP⁺ level in the brain after FR255595 treatment. FR255595 treatment had no effect on the concentration of MPP⁺ in the brain of MPTP-treated C57BL/6 mice. MPP⁺ levels in the brain at 0.5 h after MPTP treatment were 2.09 ± 0.15 and 1.05 ± 0.09 μg/g in vehicle-treated or 2.26 ± 0.11 and 1.14 ± 0.06 μg/g in FR255595 (32 mg/kg)-treated mice, respectively. Thus, there is no significant difference of MPP⁺ levels between vehicle treatment and FR255595 treatment. This result is consistent with the data above that FR255595 has no MAO-B inhibitory activity in vitro.

**Neuroprotective Action in Mouse MPTP Model**

**Brain PARP Inhibition by Treatment with FR255595.**

Because FR255595 showed potent neuroprotective effects in cultured cells, the neuroprotective properties of FR255595 were evaluated in mouse MPTP model with C57BL/6 mice. To verify the dosing of FR255595 in mouse MPTP model, PARP inhibition by FR255595 treatment in mouse brain was determined by using PARP enzyme assay. FR255595 (3.2, 10, or 32 mg/kg) was orally administered in normal mice, and nuclear extracts were prepared from mice brain at 2 h after dosing. All doses of FR255595 at 2 h markedly inhibited PAR polymer formation induced by addition of nicked DNA in PARP enzyme assay, and at 8 h, FR255595 treatment moderately but still significantly inhibited PAR polymer formation. Inhibition of PAR polymer formation was observed even at 24 h after drug treatment with the highest dosing of FR255595 (32 mg/kg) (Fig. 5).

**Detection of PARP Activation and MPTP Neurotoxicity in Mice.**

To confirm the PARP activation after MPTP intoxication in C57BL/6 mouse model, NAD contents in both the SNpc and the striatum were measured. In this assay system, NAD depletion in both SNpc and striatum was detectable after 2 h of fourth MPTP injections (Table 1). The degree of cell damage was assessed by amount of DA content and DAT binding sites in the striatum. The amount of DA content and the density of DAT binding sites were significantly decreased after 1 day of MPTP injections, and at 4 days, the reduction stabilized at about 20% of normal levels (Table 2). The depletion level of DA content was consistent with that of DAT binding sites in the striatum (Table 2) and also in the SNpc (data not shown), suggesting that measurement of DA contents in the striatum could mirror the damage in the striatum and the cell death in the SNpc.

**Effect of FR255595 with Pretreatment.**

To ascertain whether FR255595 exerts the neuroprotective effects in mouse MPTP model, FR255595 was first evaluated on the four-dose paradigm of MPTP delivery (severe model), as indicated in Fig. 6A. FR255595 was administered twice to C57BL/6 mice (3–32 mg/kg p.o.) 1 h before the first and third injections of MPTP, and after 4 days, each striatum was dissected to quantify DA and its metabolites. In this model, pretreatment of FR255595 significantly and dose dependently prevented the depletion of striatal DA, DOPAC, and HVA content (Fig. 6B) and also ameliorated the damage of...
the nigrostriatal dopaminergic terminals in the striatum, as assessed by a DAT binding assay (Fig. 6C). The depletion of DAT binding sites after MPTP intoxication and its percentage of recovery by FR255595 treatment were consistent with the degree of DA depletion and its recovery, respectively. In accordance with the last experiment (Table 1), four-dosing of MPTP again induced significant PARP activation quantified by NAD depletion in the SNpc at 4 h after the fourth injection of MPTP, with NAD levels decreased by 62.7%, and FR255595 (32 mg/kg) treatment significantly spared NAD consumption by 79.5% of normal level (Fig. 6D). These results indicate that FR255595 can also exert neuroprotective effects in vivo via PARP inhibition in accordance with in vitro data.

TABLE 1
MPTP-induced NAD reduction in SNpc and striatum
MPTP-induced NAD reduction in C57BL/6 mice brain. Following the fourth MPTP (4 × 20 mg/kg i.p. 3-h interval) injections, NAD content both in the SNpc and in the striatum were measured at 2, 4, and 24 h. In the SNpc, NAD depletion was observed after 2 h, and the reduction was significant at 4 h of last MPTP injection. In the striatum, NAD depletion was detectable even at 2 h of last MPTP injection. Values are means ± S.E.M. of n = 6 to 7 mice. **P ≤ 0.01 versus vehicle-treated control group (by one-way ANOVA followed by Dunnett’s multiple comparison test).

<table>
<thead>
<tr>
<th>Time after MPTP Treatment</th>
<th>NAD Contents</th>
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<tr>
<td></td>
<td>SNpc</td>
<td>Striatum</td>
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<tr>
<td>0h</td>
<td>11.85 ± 0.72</td>
<td>15.65 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>2h</td>
<td>9.42 ± 0.68</td>
<td>10.48 ± 0.44**</td>
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<tr>
<td>4h</td>
<td>7.43 ± 0.99**</td>
<td>9.62 ± 0.33**</td>
<td></td>
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<tr>
<td>24h</td>
<td>6.94 ± 0.81**</td>
<td>11.48 ± 0.37**</td>
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**P ≤ 0.01, statistically significant compared with control group (by one-way ANOVA followed by Dunnett’s multiple comparison test).

TABLE 2
Correlation between DA content and DAT binding site in the striatum after MPTP treatment in mice
MPTP (20 mg/kg) was injected intraperitoneally four times at 2-h intervals, and dopamine content and DAT binding site in the striatum were measured after 24 and 96 h. Both dopamine content and DAT binding site were significantly decreased by MPTP treatment after 24 h, with marked reduction after 96 h. Decrease of dopamine content level was coincident with that of DAT binding site. Values are means ± S.E.M. of n = 5 to 6 mice.

<table>
<thead>
<tr>
<th>Time after MPTP Treatment</th>
<th>DA Content</th>
<th>% of normal</th>
<th>DAT Binding Site</th>
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<tr>
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<td></td>
<td></td>
<td>0</td>
<td>0.99**</td>
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<td></td>
<td>96</td>
<td>0.99**</td>
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**P ≤ 0.01, statistically significant compared with control group (by one-way ANOVA followed by Dunnett’s multiple comparison test).

Neuroprotection by FR255595 with Post-Treatment. To determine whether the neuroprotective effect of FR255595 would also be obtained with delayed treatment, post-treatment of FR255595 after MPTP injections was conducted in C57BL/6 mice. On the two-dose paradigm of MPTP delivery (mild model; Fig. 7A), MPTP was injected twice at a 2-h interval. Administration of MPTP with this regimen resulted in mild-to-moderate reduction (approximately 40% of normal level) of striatal DA and its metabolites contents (Fig. 7B). FR255595 (32 mg/kg) was orally administered twice at 1 h and 5 h after second MPTP injections. Post-treatment of FR255595 still significantly attenuated the reduction of striatal DA and its metabolites contents in this paradigm (Fig. 7B), and also markedly attenuated neuronal damage assessed by the quantification of DAT binding sites by using brain homogenate prepared from both the striatum and the SNpc (Fig. 7C). The depletion of DAT binding sites in the SNpc was significant but relatively mild compared with that in the striatum. Thus, the depletion of DAT binding sites in the striatum was coincident with cell death in the SNpc.

Discussion
A newly synthesized PARP-1 inhibitor, FR255595, is one of the most potent candidates we have ever tested through structure-based drug design system using crystal structure of recombinant human PARP, together with high-throughput screening systems. The structure-based drug design-designed compound FR255595 and its derivatives yield no significant difference between human and rodents PARP-1 inhibitory activity, suggesting that PARP has no species differences on its active site and is consistent with a previous report that the primary structure of the enzyme is highly conserved in eukaryotes (human and mouse enzyme have 92% homology at the level of amino acid sequence), with the catalytic domain showing the highest degree of homology between different species (Mazan et al., 1989; de Murcia et al., 1994). Furthermore, more interestingly, FR255595 was more selective to PARP-1 than PARP-2 (30-fold higher selectivity) compared with nonselective general PARP inhibitors such as 3-aminobenzamide, indicating that FR255595 could be the first relatively selective inhibitor for PARP-1 among several published PARP inhibitors.

To determine PARP inhibitory properties and the neuroprotective properties of FR255595 in cultured cells, PARP activation and cell damage were induced by H2O2 exposure in both PC12 cells and SH-SY5Y cells. In this cell death system,
H$_2$O$_2$ exposure generates ROS and markedly induces PARP activation, concomitant NAD depletion, and early stage cell death. As expected given the potent PARP inhibitory activity of FR255595, this compound prevented the NAD depletion even at a low concentration and markedly attenuated cell death, even when it was added after H$_2$O$_2$ exposure. These results were consistent with a number of studies (Zhang et al., 1994; Cosi and Marien, 1998; Chiarugi et al., 2003), suggesting that PARP-1 is activated at a relatively late stage in the cell damage cascade and that delayed treatment with PARP-1 inhibitors still had potential to attenuate the cell damage involved in PARP overactivation. From the observation in this culture system, we conclude that FR255595 possesses superior neuroprotective properties through its potent PARP-1 inhibitory activity and favorable cell membrane penetration properties ($IC_{50} = 10^{-8}$ M in PARP enzyme assay versus minimum effective concentration $= 10^{-4}$ M in vitro cell death assay), compared with other PARP inhibitors such as 3-aminobenzamide ($IC_{50} = 10^{-7}$ M in PARP enzyme assay versus minimum effective concentration $= 10^{-4}$ M in vitro cell death assay) or quinoxaline derivatives of novel PARP-1 inhibitor, which we have recently identified (Iwashita A., unpublished data).

Cellular energy impairment seems to play an important part in MPTP-induced neurotoxicity. Striatal levels of NAD were decreased by 20% at 2 h after 4 times intraperitoneal injections of MPTP (20 mg/kg) in C57BL/6 mice. This partial loss of NAD seems to be selective for both the striatum (nigrostriatal dopaminergic terminal field) and the SNpc and was not observed in the cortex and hippocampus (data not shown). Here, we investigated in vivo whether MPTP acutely caused region- and time-dependent changes in brain levels of NAD, and whether such effects were modified by treatment with neuroprotective doses of FR255595. Consistent with previous reports, neurotoxic doses of MPTP caused a rapid decrease in striatal NAD, DA metabolites (Cosi and Marien, 1998), and DAT binding affinity, and these changes were ameliorated by treatment with neuroprotective doses of
FR255595, suggesting the neuroprotective actions of FR255595 on dopaminergic neurodegeneration induced by MPTP intoxication.

In the central nervous system, MPTP is oxidized to MPP+ by monoamine oxidase-B (Heikkila et al., 1984; Trevor et al., 1987), and it is taken up by dopamine neurons via the high-affinity dopamine transporter (Javitch et al., 1985). MPP+ is then concentrated in mitochondria where it inhibits complex I, which leads to superoxide anion formation. The superoxide anion reacts with nitric oxide produced by NOS to form the potent oxidant peroxynitrite, which damages intracellular proteins and DNA to cause cell death. Although PARP-1 is activated after DNA damage that mediates neuronal cell death, providing a mechanism by which PARP-1 inhibitors can exert neuroprotection, drugs that affect MPTP metabolism can also block MPTP-induced cell death. To differentiate between these mechanisms, we determined whether neuroprotection of FR255595 is achieved by inhibition of MAO-B and/or binding to DAT. FR255595 had no MAO-B inhibitory activity and no DAT binding affinity, suggesting that FR255595 is not able to affect MPTP metabolism and to inhibit the accumulation of MPTP metabolites into the cells.

It was confirmed by the present finding in vivo demonstrating that FR255595 did not affect striatal MPP+ content and MPTP-induced striatal dopamine and its metabolite content immediately after MPTP injections. The role of NOS in MPTP-induced dopaminergic neurotoxicity has been suggested by the findings on neuroprotection by nNOS inhibitors and also by the fact that mutant mice lacking nNOS gene are resistant to MPTP neurotoxicity (Schulz et al., 1995; Przedborski et al., 1996; Matthews et al., 1997; Grunewald and Beal, 1999). MPTP neurotoxicity is also attenuated by antioxidants or spin trap agents (Matthews et al., 1999). In our in vitro assay, FR255595 has no antioxidant property and NOS inhibitory activity even at a concentration of 10^{-5} M. These results suggest that FR255595 has no potential to inhibit MPTP neurotoxicity directly, and also this compound does not modulate the ROS-mediated pathway. Although the effects of FR255595 on other diverse class of known neurotransmitter receptors, ion channels, or enzymes have not been fully clarified, the potent neuroprotective properties are likely the consequence of its specific PARP-1 inhibitory activity.

As shown in the results of pharmacokinetic study,
FR255595 was orally active and the brain concentration of FR255595 at 32 mg/kg p.o. was found to be 2.73 μg/g, which is estimated as more than $10^{-4}$ M, at 2 h postdosing. This dosing regimen yielded enough brain level of FR255595 that should sufficiently exert PARP-1 inhibitory activity in the brain. As expected from its good pharmacokinetic profile, treatment with FR255595 before MPTP intoxication produced robust and significant neuroprotection. MPTP-induced PARP activation persists for some time; thus, neuroprotective effects of FR255595 in MPTP neurotoxicity presumably are related to its PARP inhibitory properties derived from relatively long-lasting higher brain concentration.

Evaluation of several PARP-1 inhibitors in a multitude of animal experiments (Cosi et al., 1996; Abdelkarim et al., 2001; Ding et al., 2001; Suh et al., 2003) has suggested that PARP inhibition therapy represents an effective approach for the treatment in a variety of diseases. The fact that PARP-1 inhibition targets a relatively late stage of oxidative cell damage should be the key of this remarkable efficacy. Therefore, the therapeutic time window of PARP-1 inhibitor could be relatively wide, as indicated by the effectiveness of post-treatment regimens in some models and also in our experiments. In this study, the post-treatment of FR255595 after MPTP intoxication showed the amelioration of neuronal damage in nigrostriatal dopaminergic pathway. This neuroprotection was observed both in the striatum and in the SNpc, suggesting that reduction of the terminal damage assessed by both DA and its metabolites contents and the amount of DAT binding sites in the striatum correlates well with the reduction of the cell death in the SNpc. In our preliminary study, we observed that the reduction of TH-positive neurons in the SNpc correlated with the decrease in DA contents in the striatum (Iwashita A., unpublished data). Thus, these results support the notion that the depletion of DAT binding sites is consistent with the loss of cell bodies in SNpc and that DAT binding assay could be the substitute of the assay to quantify the dopaminergic cell death by using TH immunostaining in MPTP-treated animal model of PD.

The neuroprotective efficacy of FR255595 in mouse MPTP model was relatively moderate, in spite of possessing potent PARP-1 inhibitory activity and high brain penetration properties in mouse brain. FR255595 is highly bound to plasma proteins (binding ratio to albumin is about 99.4%); thus, the percentage of free bodies of this compound in both serum and brain seem to be very low, perhaps decreasing the efficacy of FR255595. Alternatively, the involvement of PARP in dopaminergic neurodegeneration associated with MPTP treatment could be partial (not 100%), and there could be other independent parallel death pathways. The view is supported by the previous finding in PARP knockout animals that the recovery in dopaminergic cell death induced by MPTP was 50% (Mandir et al., 1999). Therefore, it would be important to address in the future studies in experimental animals and even in human clinical trials whether combination therapy with PARP-1 inhibitions plus the blockades of other mechanisms might augment the therapeutic efficacy given by PARP-1 inhibitors, which could delay the progression of dopaminergic neurodegeneration.

Furthermore, in our preliminary data, well known PARP-1 inhibitors such as 3-aminobenamide, 1,5-dihydroxyisouquinoline, or PJ-34 [N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide], represent mild neuroprotection (20% amelioration) even at higher doses (100–320 mg/kg i.p.), compared with that of FR255595 (50% amelioration) in the same in vivo MPTP model (Iwashita A., unpublished data), suggesting that the orally active and potent PARP-1 inhibitor FR255595, which has favorable cell membrane and brain penetration properties, could be one of the best compounds to investigate pharmacological profile after PARP-1 inhibition both in cell cultures and in animals.

In conclusion, newly synthesized PARP-1 inhibitor FR255595 exhibited potent PARP-1 inhibition both in vitro and in vivo, with significant neuroprotective activity after MPTP intoxication in mice, suggesting that this compound could be not only an important tool for investigation of the physiological role of PARP-1 in neurodegeneration but also an attractive therapeutic candidate for neurodegenerative disorders such as Parkinson’s disease.

Acknowledgments

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References


Ding Y, Zhou Y, Lai Q, Li J, Gordon V, and Diaz FG (2001) Long-term neuroprotection with the orally active and potent PARP-1 inhibitor FR255595, which has favorable cell membrane and brain penetration properties, could be one of the best compounds to investigate pharmacological profile after PARP-1 inhibition both in cell cultures and in animals.

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