JPET Fast Forward. Published on February 25, 2004 as DOI: 10.1124/jpet.103.064642


# 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 MPTP Model of Parkinson's Disease

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**Running title:** PARP inhibitor FR255595 on MPTP-induced neurodegeneration

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No. of text pages: 39

No. of tables: 2

No. of figures: 7

No. of references: 40

No. of words in the Abstract: 250

No. of words in the Introduction: 564

No. of words in the Discussion: 1558

**Recommended section:** Neuropharmacology

**Abbreviations:**

PARP, poly(ADP-ribose) polymerase; NAD, nicotinamido adenine dinucleotide; FR255595, 2-{3-[4-(4-chlorophenyl)-1-piperazinyl]propyl}-4(3H)-quinazolinone; $H_2O_2$, hydrogen peroxide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; DAT, dopamine transporter; MAO-B, monoamine oxidase-B; TBARS, thiobarbituric acid reactive substances; DA, dopamine; DOPAC, dihydroxyphenylacetic acid ; HVA, homovanillic acid ; SNpc, substantia nigra pars compacta; ROS, reactive oxygen species; ANOVA, analysis of variance.

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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 FR255595 (2-{3-[4-(4-chlorophenyl)-1-piperazinyl] propyl}-4(3H)-quinazolinone) 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 $IC_{50}$ value of 11nM and was orally active and highly brain penetrable. Here we show that prevention of PARP activation by FR255595 protects against both ROS-induced cells injury *in vitro* and 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 ($10^{-9}$ M to $10^{-5}$ M) significantly reduced PARP activation and cell death. In mice MPTP model, MPTP (20mg/kg, i.p.) intoxication lead to PARP activation and cell damage in the nigro-striatal 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.

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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 nicotinamide adenine dinuleotide (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 (AIF) (Yu et al., 2002). Translocation of AIF from the mitochondria to the nucleus is dependent on PARP activation in neurons treated with various DNA-damaging stimuli such as N-methly-N'-nitro-N-nitrosoguanidine (MNNG), 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 such as Parkinson's disease (PD), a chronic progressive neurologic 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 nigro-striatal dopaminergic pathway and produce parkinsonism in humans and in experimental animals (Kopin and Markey, 1988; Langston, 1996; Prezedborski 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 (NO)-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 noradrenalin 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 postmortem 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 *in vitro* experimental neuronal cell death models, in which PARP-1 is markedly activated by $H_2O_2$ exposure. Finally, the neuroprotective properties of FR255595 were evaluated in a mouse MPTP model which is a pertinent *in vivo* model of PD.

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## Materials and Methods

### Materials

Rat pheochromocytoma PC12 cells and human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA). FR255595 (2-{3-[4-(4-chlorophenyl)-1-piperazinyl] propyl}-4(3H)-quinazolinone; 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 (St. Louis, MO) and tissue culture dishes were from NUNC (Taastrup, Denmark). Hydrogen peroxide (30%), MTT was from Wako Chem. (Tokyo, Japan). Recombinant human PARP enzyme was purchased from Trevigen, 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 (St. Louis, MO).

### Measurement of PARP inhibitory activity *in vitro*

To assess the PARP-1 or PARP-2 inhibitory activity of FR255595, PARP activity was evaluated as previously described (Banasik et al., 1992) with minor modifications. PARP enzyme assay was carried out in a final volume of 100µl consisting of 50mM Tris-HCl (pH8.0), 25mM $MgCl_2$, 1mM dithiothreitol, 10µg activated salmon sperm DNA, 0.1µCi 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 FR255595. The reaction mixture was incubated at room temperature (23°C) for 15min, and the reaction was terminated by adding 200µl of ice cold 20% trichloroacetic acid (TCA) and incubated at 4°C for 10min. The precipitate

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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, the published methods were used as previously described with minor modifications (Lahiri DK and Ge Y, 2000). To prepare the nuclear extracts from the cultured cells, two million cells cultured in F25 flask were washed with 10mL of PBS (phosphate buffered saline) and cells were re-suspended in 500μL of cold buffer A (10mM HEPES pH7.6, 15mM KCl, 2mM $MgCl_2$, 0.1mM EDTA, 0.1% NP40) and homogenized gently. The homogenate was centrifuged at 5000g for 30 second and the supernatant containing cytoplasm and RNA was removed. The nuclear pellet was re-suspended in 50μL of ice-cold buffer B (50mM HEPES pH7.9, 400mM KCl, 0.1mM EDTA, 10% Glycerol). The tube was mixed thoroughly on a micro tube mixer for 15 min at 4°C. The nuclear extract was centrifuged at 11000g 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 brain tissue per 1mL and ten 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 300μL of buffer B was added to the nuclear pellet in each of the Eppendorf tubes. The tubes

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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.

**Determination of MAO-B inhibitory activity**

MAO-B activity in mouse brain was determined by radio-assay as described by Wurtman and Axelrod (1963) with minor modifications using [$^{14}$C]2-β-ethyl-1-phenylethylamine HCl (PEA, about 50mCi/mmol;NEN) as substrates at concentrations of 20.8μM (specific activity 1.6mCi/mmol). Briefly, mitochondria homogenate from mouse brain in 85μl of 67mM sodium phosphate buffer (pH 7.4) and 5μl of desired concentrations ($10^{-8}$ to $10^{-5}$M) of the compounds was pre-incubated in 96 well plates at 37°C for 10min. Following the pre-incubation, 10μl of [$^{14}$C] 2-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 100μl of cold 3M HCl. The deaminated products were extracted by 0.5mL Ethyl acetate and the separation of the two phases was facilitated by centrifugation (7000g) at 15° for 10 min. The samples were analyzed in the scintillation counter for the amount of $^{14}$C in the upper phase.

**DAT binding assay**

To determine the affinity of FR255595 to dopamine transporter (DAT), and also to evaluate the level of cell damage after MPTP intoxication in mice, the striatum and/or substantia nigra were dissected from mice brain and homogenate with KRH buffer containing 125mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 1.3mM $CaCl_2$, 1.2mM

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$KH_2PO_4$, 5.6mM glucose, 0.01mM nialamide, 25mM 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 membranes preparation from the striata or substantia nigra, about 125μg of protein, to an incubation solution (KRH buffer) containing 5 nM [$^3$H]-mazindol (NEN/Dupont; 17Ci/mmol) and 300nM desipramine to occlude binding to the NE transporter. Samples in duplicate were incubated at 4°C for 1hr and the reaction was stopped by addition ice-cold KRH buffer. The reaction solution was rapidly filtrered through Whatman GF/C filters and three washes with binding buffer using a cell harvester. Nonspecific binding was determined in the presence of 10μM of benztropine or cocaine.

**Determination of radical scavenging activity**

For measurement of lipid peroxidation, TBARS (Thiobarbituric acid reactive substances) were estimated using the modified method of Buege and Aust (1978) and Callaway et al. (1998). Briefly, mice brain synaptosomes were prepared from C57/BL6 mouse (from Japan SLC, Shizuoka, Japan). To evaluate the inhibitory activity of FR255595, different concentrations of the compounds was dissolved 50% dimethyl sulfoxide (DMSO), and then 5μl were added to each rat brain synaptosome and incubated with ammonium ferric sulfate (100 μM) at 37°C for 30min. The reaction was stopped with addition of 20% TCA, and the precipitated proteins were removed by centrifugation at 10,000g for 15min. The aliquots of supernatant were then added to an equal volume of thiobarbituric 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 (MDA).

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Percent inhibition of TBARS production was calculated as follows: % inhibition =[(Max - Drug)/(Max - Base)] x 100, where Max is the values in the presence of ammonium ferric sulfate, Base is the values in the absence of ammonium ferric sulfate and Drug is the values of test compounds.

**Determination of NOS inhibitory activity**

NOS catalytic activity was assayed by measuring the $Ca^{2+}$-dependent conversion of [$^3$H]-arginine to [$^3$H]-citrulline as described by Huang et al. (1993). For this assay, dissected mouse brain was homogenated in 20 vol (wt/ vol) of 25mM Tris buffer (pH7.4) containing 1mM EDTA and 1mM EGTA. After centrifugation (20,000 x g for 15 min at 4°C), 25 µl of supernatant was added to 75 µl of 50mM Tris buffer (pH 7.4) containing 1mM NADPH, 1mM EDTA, 3mM $CaCl_2$, and 0.1µCi of [$^3$H] arginine (specific activity 64Ci/mmol;NEN) in the absence or presence of FR255595 solution and incubated for 15 min at 37°C. The reaction was terminated by the addition of 250µl Dowex AG50WX-8 (Pharmacia) and cooled on ice. After centrifugation, [$^3$H]citrulline was quantified by liquid scintillation counting of 100 µl supernatant. No significant [$^3$H]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% $CO_2$ at 37°C for 24hr. For all experiment, cells were seeded at a density of 4x10$^4$cells/well in 96 well culture plates and allowed to attach overnight. The cells were incubated with or without hydrogen peroxide (100 or

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300μM) and FR255595 for 6hr. To evaluate cell survival, MTT assay was performed according to a minor modification of the original method (Mosmann, 1983). Briefly, MTT [3-(4, 5-demethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide] was added to the cultures at a final concentration of 0.2 mg/ml and after incubation at 37°C for 2 hr, the media was removed carefully and the reaction was stopped by addition of isopropanol containing 0.04N HCl. The absorbance of each well was measured at 590 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

***Drug treatment:*** FR255595 was dissolved in 100% DMSO at $10^{-2}$ M and then diluted in DMEM without serum. This solution was added to culture plate 1hr before $H_2O_2$ exposure.

***Determination of cellular NAD level:*** To determine NAD level in cultured cells, PC12 cells were seeded at $2x10^5$ cell / well in 24 well plates and cultured for 24hrs. FR255595 was added to cell media at several concentrations. Thirty min later, cells were exposed 100μM $H_2O_2$ for 30min and cells were detached using by cell scraper and then collected in microcentrifuge tube by centrifugation for 5 min x 100 g at 4°C. To quantify the NAD level in mouse brain, brain homogenate (10 mg tissue/ 150μl in PBS) dissected from the striatum and the substantia nigra were prepared, respectively. Cells or brain homogenate were extracted with 200μL of 0.5M $HClO_4$ for 15 min, and then 60μL of 2M KOH/0.2M $K_2HPO_4$-$KH_2PO_4$ pH7.5 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 $2x10^6$ cells in F75 culture flask and cultured for 24hr. Then FR255595 was added to culture media at several concentrations. 30 min later, cells were exposed 100μM $H_2O_2$

for 30min and cells were detached using cell scraper and then prepared nuclear extracts as described above. PARP activation was determined by PARP-1 enzyme assay using nuclear extracts as a mixture of PARP-1 enzyme and nicked DNA.

***Poly (ADP-ribose) Polymer Western blots:*** Western blot analysis of Poly (ADP-ribose) polymer was performed using PC12 cells lysates. Samples containing 20mg protein were loaded, separated on a 5-20% of polyacrylamide gel and transferred to a nitrocellurose 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 non-fat 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 MPTP model, 9 to 10 week old male C57BL/6 mouse weighing 19-22 g from 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-hour light/dark cycle (light on at 07:00). 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. 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

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properties of FR255595 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 another is the two-dose paradigm of that to induce milder cell injury. The animals were received 4 (severe model) or 2 (mild model) x 20mg/kg MPTP with i.p. injection at 2h interval. FR255595 suspended in 0.5% methylcellulose was administered at 3.2 to 32mg/kg p.o. twice at 1h prior to first injection of MPTP and at 1h prior to third injection of MPTP at 4hr interval for severe model. In the mild model, FR255595 suspended in 0.5% methylcellulose was administered at 3.2 to 32mg/kg p.o. twice at 1h following fourth dose of MPTP and at 4hr later. The administration volume was adjusted to 10 mL/kg in all experiments.

***Preparation of SN and striatum homogenate:*** For NAD assay and DAT binding assay used by tissue homogenates, whole brains were dissected and put on ice-cold brain slicer immediately. A 1mm thick slice including SNpc and whole bilateral striata 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 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 mice brain:*** To evaluate the inhibition of PARP-1 activity in the brain following the treatment of FR255595, FR255595 was administered in normal C57BL/6 mice at 3.2 to 32mg/kg p.o. once, and each brain was dissected at different time point (2, 8, 24hr) 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 HPLC with electochemical detection. Four days after drug administration, brains were quickly removed and the striatum were 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 0.1M perchloric acid and 0.1mM EDTA-2Na containing 50ng/mL isoproterenol as internal standard. After centrifugation (15000xg, 15min, 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 catecholeamine column (SL-5ODS 3.0 x 150mm, Eicom Co.,Ltd., Kyoto, Japan) with ECD-300 electrochemical detector (+450mV). The mobile phase consisted of 0.1M sodium acetate buffer, 0.1M citrate buffer, pH 3.5, 0.02mM EDTA, 1mM sodium octane sulfonic acid and 15% methanol. The flow rate was maintained at 0.5ml/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 following p.o. administration at 32mg/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 0.5h and 2hr after dosing, and the plasma and brain level of FR255595 were measured using high-performance liquid chromatography (HPLC). For measurement of $MPP^+$ levels in the brain, FR255595 (32mg/kg) was administered orally, and 1hr after dosing, MPTP (20mg/kg, i.p.) was administered. The brain was collected at 0.5h and 2hr after dosing, and $MPP^+$ level in the brain was measured by HPLC. A 200μl of plasma sample was extracted with 400μl

acetonitrile and the organic phase was separated. Whole brain was homogenized in 1ml saline and the 200µl was further homogenized in 400µl of acetonitrile solution. Each supernatant subsequently evaporated under a gentle stream of nitrogen and the dried residue was reconstituted in 150µl of 50% acetonitrile. The 35µl volume of reconstitute was injected onto a reverse phase column Inertsil ODS-3, 4.5mm x 150mm, i.d. 5µm (GL Science, Japan) and detected using a Waters Model 486 tunable absorbance detector in UV mode at $\lambda_{max}$ of 225nm. The mobile phase consisted of 40% v/v acetonitrile in 20mM $KH_2PO_4$ buffer.

**Statistical analysis**

The $IC_{50}$ 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.

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## 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 activation following 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 $IC_{50}$ of 11± 0.4 nM, 13 ± 0.6 nM and 12 ± 0.5 nM in human, rat and mouse PARP, respectively (Fig.2A). Furthermore, FR255595 was shown to be more selective to PARP-1 ($IC_{50}$ = 11nM) than PARP-2 ($IC_{50}$ = 300nM) compared with non-selective general PARP inhibitor such as 3-aminobenzamide ($IC_{50}$ = 10μM for PARP-1 vs $IC_{50}$ = 10μM for PARP-2).

### Specificity of FR255595

A number of drugs which 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 ($10^{-8}$ to $10^{-5}$M) did not affect deamination of the MAO-B substrate PEA in rat brain homogenate even if applied with pre-incubation (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 [$^3$H]-mazindol as a

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radioligand. In this assay, FR255595 had no potential to inhibit mazindol binding to DAT even at a concentration of $10^{-5}$M, whereas nomifensine which was used as positive control inhibited at the 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 [$^{3}$H]-citrulline production in the concentration ranging from $10^{-7}$ M to $10^{-5}$ M, although FR255595 had no inhibitory activity up to $10^{-5}$M (Fig. 2E).

**Neuroprotective action in PC12 cells and SH-SY5Y cells**

In this study, we first confirmed whether $H_2O_2$ 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 $^{32}$P-NAD polymer and by detection of poly (ADP-ribose) polymer (PAR polymer) formation using western blot analysis in PC12 cells. 100μM $H_2O_2$ exposure for 30 min markedly increased incorporation of $^{32}$P-NAD polymer (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 $^{32}$P-NAD polymer and PAR polymer formation as shown in Fig.3A and in lane 3 of Fig.3B, respectively. PARP activation by $H_2O_2$ 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 ($10^{-9}$M to $10^{-5}$M). Exposure of $H_2O_2$ for 6hr induced severe cell damage, although FR255595 treatment at a concentration

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range from $10^{-8}$M to $10^{-5}$M significantly attenuated cell death (Fig.3D).

In SH-SY5Y cells, $H_2O_2$ -induced PARP activation and NAD depletion were also observed (data not shown), and treatment with FR255595 ($10^{-9}$M to $10^{-5}$M) 30 min before exposure of $H_2O_2$ significantly prevented the NAD reduction and neuronal cell death (Fig. 4A). This neuroprotective effect was also observed when FR255595 was added even after the onset of PARP activation (2hr after $H_2O_2$ exposure, Fig. 4B).

**Pharmacokinetic study in mice**

The plasma and brain concentrations of FR255595 were determined at 0.5 hr and 2 hr following oral administration at a dose of 32 mg/kg in C57BL/6 mice. Mean plasma and brain concentration was 2.37μg/g and 6.54μg/g at 0.5hr, 1.69μg/g and 2.73μg/g at 2hr, 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 (0.5h after dosing).

**$MPP^+$ level 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 following 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 0.5h and 2h 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 (32mg/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*.

JPET Fast Forward. Published on February 25, 2004 as DOI: 10.1124/jpet.103.064642
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**Neuroprotective action in mouse MPTP model**

***Brain PARP inhibition by treatment with FR255595:*** As FR255595 showed potent neuroprotective effects in cultured cells, 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 2hr after dosing. All doses of FR255595 at 2hr markedly inhibited PAR polymer formation induced by addition of nicked DNA in PARP enzyme assay, and at 8hr, FR255595 treatment moderately but still significantly inhibited PAR polymer formation. Inhibition of PAR polymer formation was observed even at 24hr after drug treatment when the highest dosing of FR255595 (32mg/kg) was conducted (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 2hr of fourth MPTP injections (Table 1). The degree of cell damage was assessed by amount of DA contents and DAT binding sites in the striatum. The amount of DA contents 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 contents 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.

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***Effect of FR255595 with pre-treatment:*** To ascertain whether FR255595 exerts the neuroprotective effects in mouse MPTP model, FR255595 was firstly evaluated on the four-dose paradigm of MPTP delivery (severe model), as indicated in Figure 6A. FR255595 was administered twice to C57BL/6 mice (3.2 to 32mg/kg po) 1h prior to first and third injections of MPTP, and after 4 days, each striatum was dissected to quantify DA and its metabolites. In this model, pre-treatment of FR255595 significantly and dose-dependently prevented the depletion of striatal DA, DOPAC and HVA contents (Fig. 6B), and also ameliorated the damage of the nigrostriatal dopaminergic terminals in the striatum, assessed by DAT binding assay (Fig. 6C). The depletion of DAT binding sites following MPTP intoxication and its % recovery by FR255595 treatment was consistent with the degree of DA depletion and its recovery, respectively. In accordance with the last experiment (shown in Table 1), four-dosing of MPTP again induced significant PARP activation quantified by NAD depletion in the SNpc at 4hr following fourth injection of MPTP and NAD level was decreased by 62.7% of normal level, FR255595 (32mg/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.

***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 following 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 2hr 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 (32mg/kg) was orally administered twice at 1hr and 5hr following second MPTP injections. Post-treatment of FR255595 still

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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.

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## Discussion

A newly synthesized PARP-1 inhibitor FR255595 is one of the most potent candidates we have ever tested through structure-based drug design (SBDD) system using crystal structure of recombinant human PARP, together with high-throughput screening systems. The SBDD-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 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 (Mazen et al., 1989; de Murcia et al., 1994). Furthermore, more interestingly, FR255595 was more selective to PARP-1 than PARP-2 (30-folds higher selectivity) compared with non-selective general PARP inhibitor 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 $H_2O_2$ exposure in both PC12 cells and SH-SY5Y cells. In this cell death system, $H_2O_2$ exposure generates ROS and induces the PARP activation markedly, concomitant NAD depletion and early-stage cell death. As expected given the potent PARP inhibitory activity of FR255595, this compound completely prevented the NAD depletion even at a low concentration and markedly attenuated cell death, even when it was added following $H_2O_2$ exposure. These results were consistent with a number of studies (Zhang et al., 1994; Cosi and Marien, 1998; Chiarugi et al., 2003) suggesting

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that PARP-1 is activated at a relatively late stage in the cell damage cascade, and that delayed treatment of PARP-1 inhibitor still had a potential to attenuate the cell damage involved in PARP overactivation. From the observation in this culture system, we conclude that FR255595 represents 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 vs MEC = $10^{-8}$M in *in vitro* cell death assay ), when compared with other PARP inhibitors such as 3-aminobenzamide ($IC_{50} = 10^{-5}$M in PARP enzyme assay vs MEC = $10^{-4}$M in *in vitro* cell death assay) or quinoxaline derivatives of novel PARP-1 inhibitor, which we have recently identified (Iwashita et al., unpublished data).

Cellular energy impairment appears to play an important part in MPTP-induced neurotoxicity. Striatal levels of NAD were decreased by 20% at 2hr after 4 times intraperitonial injections of MPTP (20mg/kg) in C57BL/6 mice. This partial loss of NAD appeared 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 level 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 it was shown that 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 following 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 if 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 $MPP^+$ contents 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 (Grunewald and Beal, 1999; Matthews et al., 1997; Przedborski et al., 1996; Schulz et al., 1995). 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. While the effects of FR255595 on other diverse class of known neurotrasmitter receptors, ion channels or enzymes have not fully clarified, the potent neuroprotective properties are likely the

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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 $10^{-6}$M, at 2hr post dosing. This dosing regimen yielded enough brain level of FR255595 that should sufficiently exerts PARP-1 inhibitory activity in the brain. As expected from its good pharmacokinetic profile, treatment with FR255595 prior to 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 (Abdelkarim et al., 2001; Cosi et al., 1996; 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 following 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

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decrease in DA contents in the striatum (Iwashita et al., unpublished data). Thus, these results support the notion that the depletion of DAT binding sites is consistent with the loss of cell body in SNpc, and DAT binding assay could be the substitute of the assay to quantify the dopaminergic cell death by using TH immunonostaining 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 mice 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 quite 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 demonstrating that the recovery in dopaminergic cell death induced by MPTP was 50% in the animals (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-aminobenzamide, DHIQ or PJ-34, represent mild neuroprotection (20% amelioration) even at higher doses (100 to 320mg/kg, i.p.), compared with that of FR255595 (50% amelioration) in the same *in vivo* MPTP model (Iwashita et al., unpublished data), suggesting that orally active and potent PARP-1 inhibitor

FR255595, which has favorable cell membrane and brain penetration properties, could be one of the best compound to investigate pharmacological profile after PARP-1 inhibition both in cultured cells and in animals.

In conclusion, newly synthesized PARP-1 inhibitor, FR255595, exhibited potent PARP-1 inhibition both in *in vitro* and *in vivo*, with significant neuroprotective activity following 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.

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**Acknowledgments**

We thank Dr. Raymond D. Price for his helpful comments in preparing the manuscript.

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JPET Fast Forward. Published on February 25, 2004 as DOI: 10.1124/jpet.103.064642
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## Figure legends

Figure 1

Chemical structure of FR255595 2-{3-[4-(4-chlorophenyl)-1-piperazinyl]propyl}-4(3H)-quinazolinone

Figure 2

PARP-1 inhibitory activity of FR255595 in human recombinant PARP, rat PARP-1 and mouse PARP-1 from nuclear extracts of rat and mouse brain, respectively. $IC_{50}$ values (mean ± S.E.M.) were calculated from the concentration dependence of the inhibition curves by using computer-assisted non-linear regression analyses (A). Comparison of inhibitory activities between FR255595 and positive control for pargyline in the MAO-B assay (B), for nomifensine in the DAT binding assay (C), for vitamin E in the TBARS assay (D) and 7-nitroindazole in the NOS assay (E). FR255595 did not exhibit MAO-B inhibitory activity, DAT affinity, radical scavenging activity and NOS inhibitory activity at a concentration from $10^{-8}$ to $10^{-5}$M in all assays. Values are means ± S.E.M. of n=3 determinations tested in triplicate.

Figure 3

Hydrogen peroxide-induced PARP activation, NAD depletion and cell death was prevented by PARP inhibition with FR255595 treatment in PC12 cells. Exposure of 100μM $H_2O_2$ for 30 min induced marked PARP activation evaluated by the incorporation of $^{32}$P-NAD polymer and treatment with FR255595 (10μM) completely inhibited PARP activation (A). PARP activation was also detected by using western blot after $H_2O_2$ exposure (100μM) for 30 minutes in PC12 cells. PAR polymer formation in the vehicle treated group was under detectable level (lane 1), however, $H_2O_2$

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exposure induced marked PAR polymer formation represented as 116 kDa protein (lane 2). This PAR polymer formation was completely prevented by addition of $10^{-5}$M FR255595 (lane 3). This photograph is representative of three analyses (B). FR255595 at concentrations from $10^{-9}$M attenuated NAD depletion, and at a $10^{-5}$M FR255595 prevented NAD depletion completely (C). Exposure of 100μM $H_2O_2$ for 6hr produced severe cell damage evaluated by MTT assay. This damage was significantly reduced by addition of $10^{-8}$ to $10^{-5}$M FR255595 0.5hr prior to $H_2O_2$ 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).

Figure 4

FR255595 protected against $H_2O_2$ cytotoxicity in neuroblastoma cell line SH-SY5Y. Exposure of 300μM $H_2O_2$ for 4hr produced severe cell damage evaluated by MTT assay. Neuronal cell death was significantly attenuated by pre-treatment (0.5hr prior to $H_2O_2$ exposure) of FR255595 at a concentration from $10^{-9}$M to $10^{-5}$M (A). Furthermore, post-treatment (2hr after $H_2O_2$ exposure) of FR255595 at a concentration from $10^{-8}$M to $10^{-5}$M also significantly reduced cell death (B). Data are presented as 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).

Figure 5

The ratio of PARP-1 inhibition by oral administration of FR255595 was evaluated in

normal mice brain. Brain nuclear extracts from normal or treated C57mice (3.2, 10 or 32mg//kg) were prepared at 2, 8 and 24hr after treatment. Extracts containing PARP enzyme and drug were used for *in vitro* PARP-1 assay to evaluate the PARP-1 inhibition level. Until 8hr after treatment of FR255595, all doses of this compound significantly inhibited PARP activation induced by addition of nicked-DNA in *in vitro* assay system. At 24hr, only the highest dose of FR255595 (32mg/kg) slightly but significantly inhibited PARP activation in this assay system. Values are means ± S.E.M. of n=6-7 mice. *, $P<0.05$, **, $P<0.01$ versus vehicle treated control group (by one-way ANOVA followed by Dunnett's multiple comparison test).

Figure 6

Effects of FR255595 on MPTP-induced depletion of striatal DA, DOPAC and HVA contents. MPTP was intraperitoneally injected four times at 2hr intervals and FR255595 was administered twice by p.o. at 1hr prior to first and third injection of MPTP. The striatum were dissected from each mouse at 4 days after MPTP intoxication and then DA, DOPAC and HVA were measured by HPLC with electrochemical detection. DAT binding assay was conducted using the same samples. (A) Schematic representations of the mouse MPTP model with a four-dose paradigm of MPTP delivery and pre-treatment of FR255595. (B) FR255595 dose-dependently attenuated the depletion of dopamine and its metabolite contents, and the effect of FR255595 was significant at a dose of 10 and 32 mg/kg. (C) FR255595 dose-dependently inhibited the depletion of DAT biding sites. Neuroprotective efficacy and potency of FR255595 against MPTP neurotoxicity was coincident with B. (D) FR255595 at a dose of 32mg/kg significantly spared NAD consumption in the SNpc 4hr following fourth injection of MPTP. Data are presented as mean ± S.E.M. of 6-8

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mice. Significant difference from control value are indicated by * for P<0.05, ** for P<0.01 (by one-way ANOVA followed by Dunnett's multiple comparison test) or by ## for P<0.01(by Student's t-test).

Figure 7

Post-treatment of FR255595 also attenuated neurotoxicity in the two-dose paradigm of MPTP delivery. FR255595 was orally administered at a dose of 32mg/kg after MPTP (20mg/kg x 2, i.p.) intoxication. (A) Schematic representations of the mouse MPTP model with a two-dose paradigm of MPTP delivery and post-treatment of FR255595. (B) FR255595 significantly attenuated depletion of dopamine and its metabolites contents. (C) The neuroprotective effect was confirmed by using DAT binding assay in both striatum and SNpc. Data are presented as mean ± S.E.M. of 6-7 mice. Significant difference from control value are indicated by ** P<0.01, statistically significant compared with control group (by Student's t-test).

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TABLE 1

**MPTP-induced NAD reduction in SNpc and Striatum**

| Time after MPTP treatment | NAD contents (nmol / mg wet weight) | |
|---|---|---|
| | SNpc | Striatum |
| 0h | 11.85 ± 0.72 | 15.65 ± 0.52 |
| 2h | 9.42 ± 0.68 | 10.48 ± 0.44 ** |
| 4h | 7.43 ± 0.99 ** | 9.62 ± 0.33 ** |
| 24h | 6.94 ± 0.81 ** | 11.48 ± 0.37 ** |

MPTP induced NAD reduction in C57BL/6 mice brain. Following the fourth MPTP (4 x 20mg/kg, i.p., 2hr interval) injections, NAD contents both in the SNpc and in the striatum were measured at 2, 4 or 24hr. In the SNpc, NAD depletion was observed after 2hr and the reduction was significant at 4hr of last MPTP injection. In the striatum, NAD depletion was detectable even at 2hr of last MPTP injection. Values are means ± S.E.M. of n=5-6 mice. ** P<0.01, statistically significant compared with control group (by one-way ANOVA followed by Dunnett's multiple comparison test).

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TABLE 2

**Correlation between DA content and DAT binding site in the striatum after MPTP treatment in mice**

| Time after MPTP treatment | DA content (% of Normal) | DAT binding site (% of Normal) |
|---|---|---|
| **0h** | **100 ± 4.4** | **100 ± 4.8** |
| **24h** | **36.8 ± 4.2 **** | **39.6 ± 2.7 **** |
| **96h** | **19.7 ± 2.4 **** | **15.9 ± 1.8 **** |

Comparison with dopamine content and DAT biding site in the striatum following MPTP intoxication in C57BL/6 mice. MPTP (20mg/kg) was injected intraperitoneally four times at 2hr intervals and dopamine content and DAT binding site in the striatum were measured after 24hr and 96hr. Both dopamine content and DAT binding site were significantly decreased by MPTP treatment after 24hr, with marked reduction after 96hr. Decrease of dopamine content level was coincident with that of DAT binding site. Values are means ± S.E.M. of n=5-6 mice. ** P<0.01, statistically significant compared with control group (by one-way ANOVA followed by Dunnett's multiple comparison test).

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Fig.1 Iwashita et al

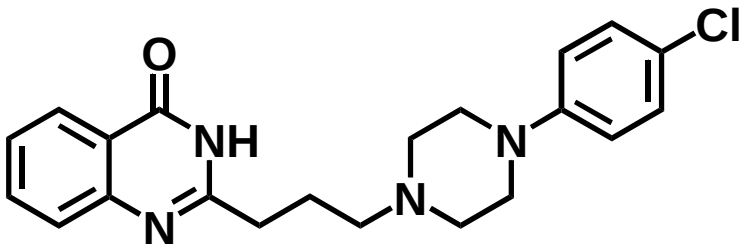

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Fig.2 Iwashita et al

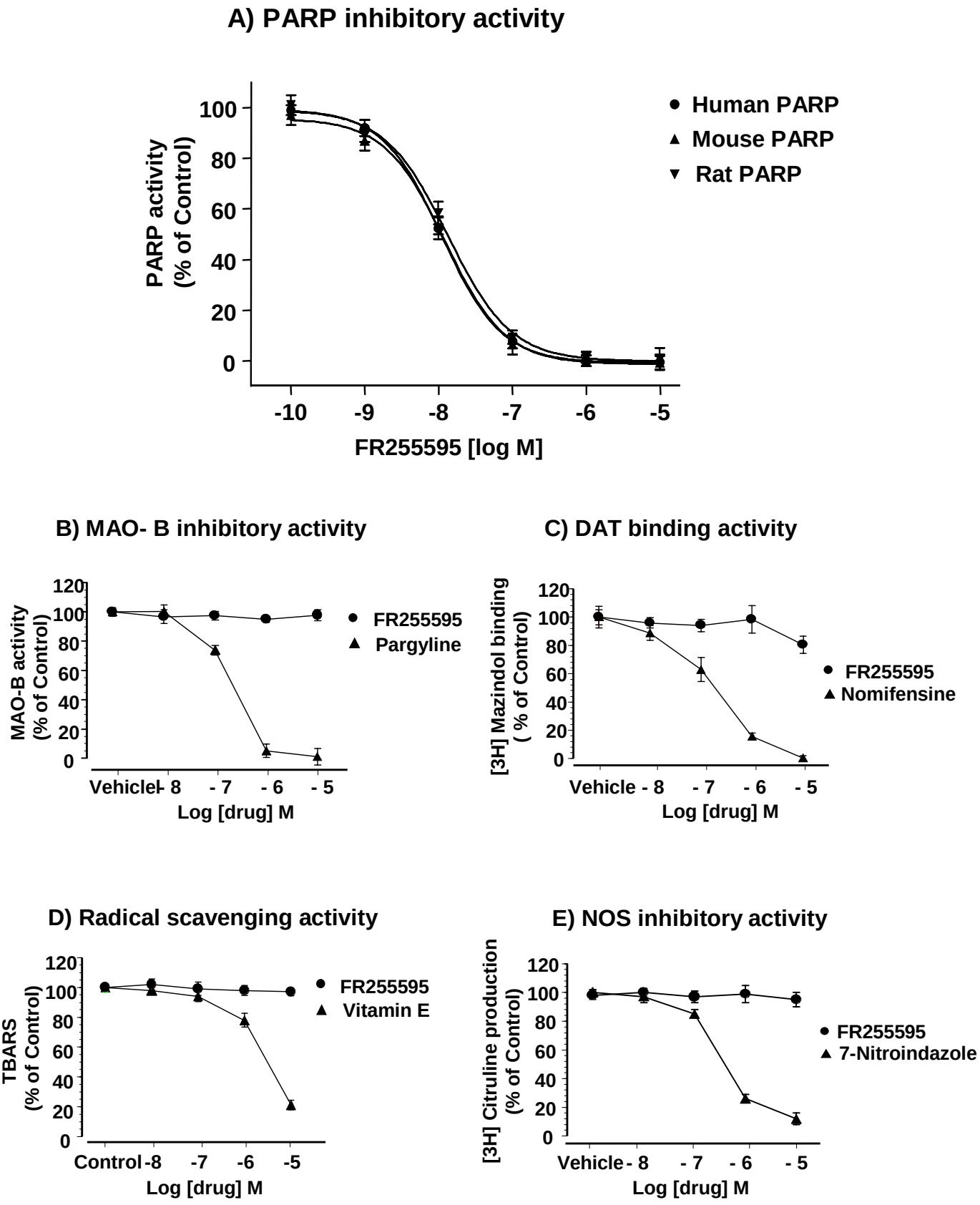

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Fig.3 Iwashita et al

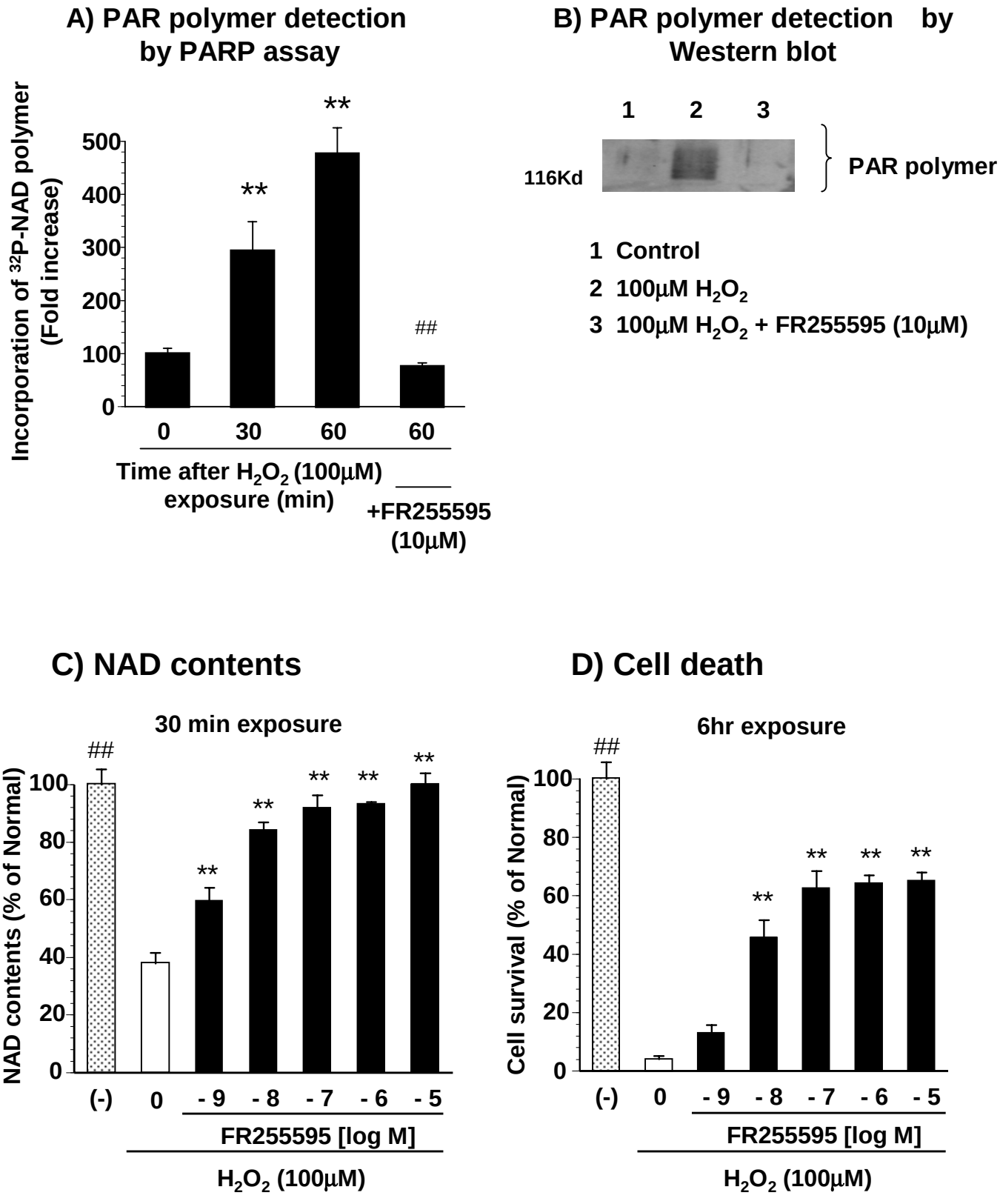

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Fig.4 Iwashita et al

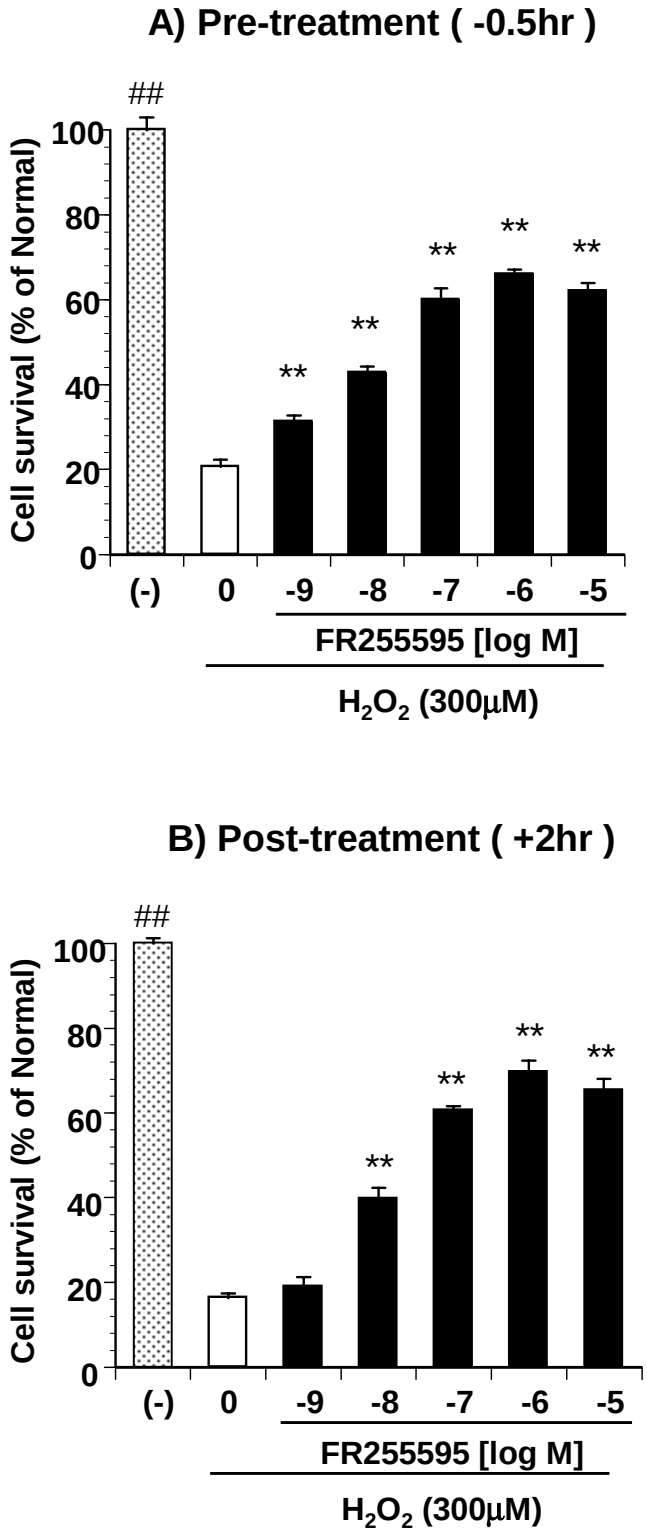

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Fig.5 Iwashita et al

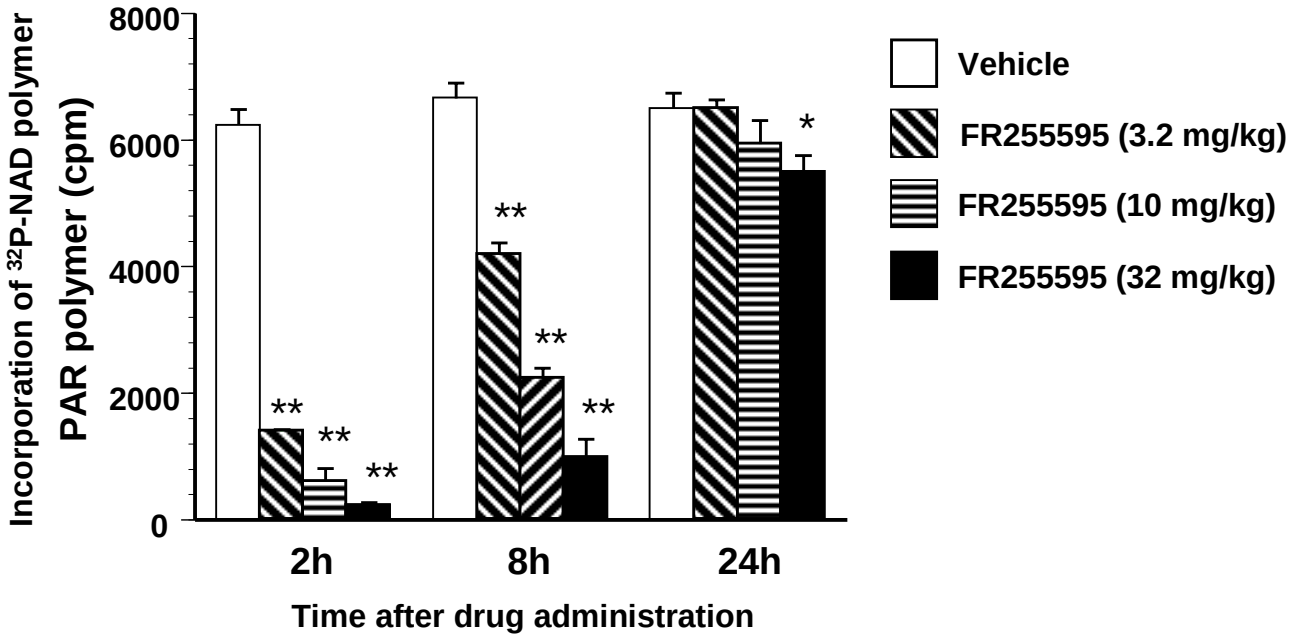

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Fig.6 Iwashita et al

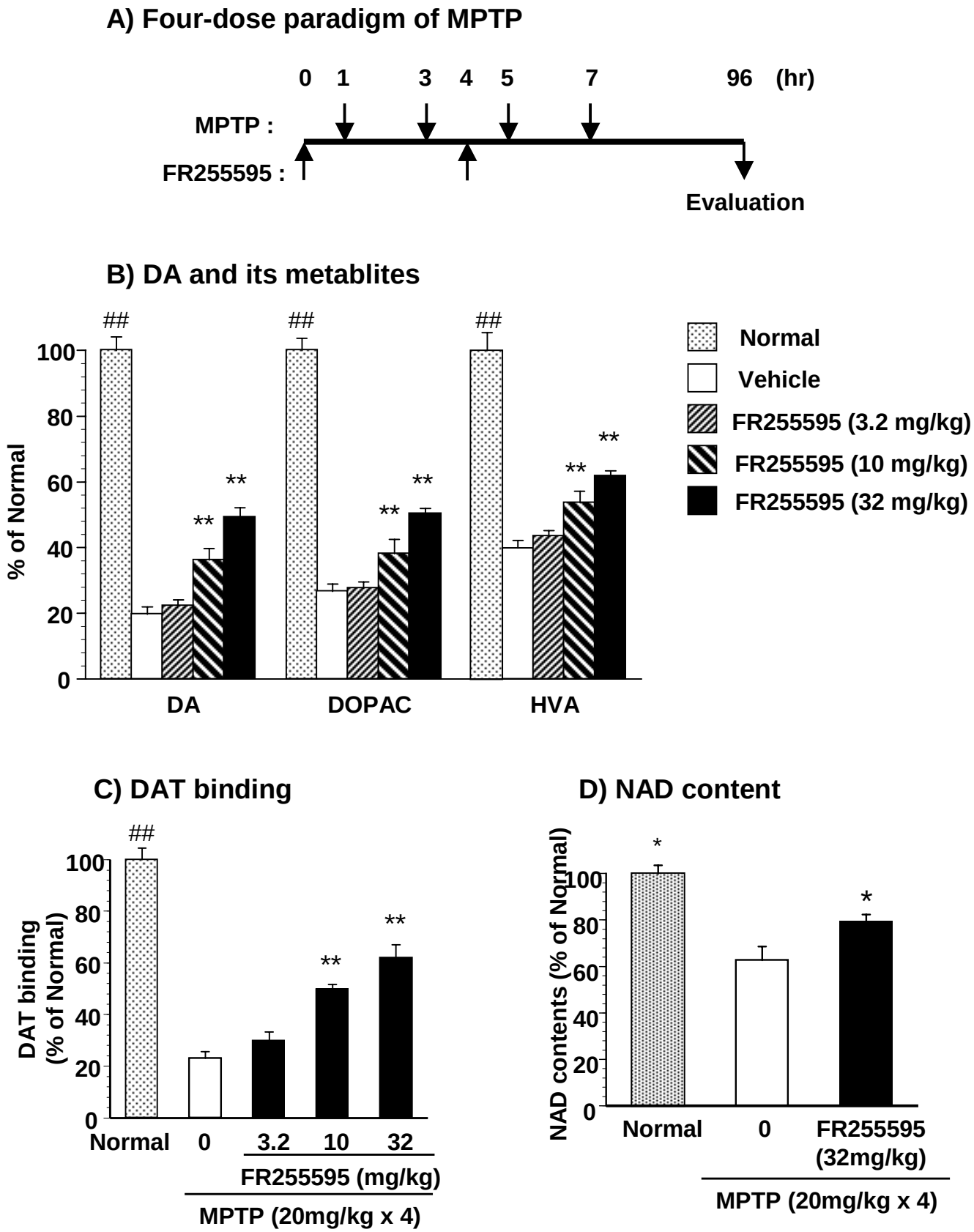

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Fig.7 Iwashita et al