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A New Poly (ADP-ribose) Polymerase Inhibitor, 2-(4-chlorophenyl)-5-quinoxalinecarboxamide (FR261529), Ameliorates Methamphetamine-Induced Dopaminergic Neurotoxicity in Mice

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Running title: PARP and neurotoxicity by methamphetamine

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Abbreviations:

PARP, poly (ADP-ribose) polymerase; METH, methamphetamine; DA, dopamine; FR261529, (2-(4-chlorophenyl)-5-quinoxalinecarboxamide; ROS, reactive oxygen species; PD, Parkinson's disease; NAD, nicotinamide adenine dinuleotide; SNpc, substantia nigra pars compacta; DAT, dopamine transporter; MAO, monoamine oxidase; nNOS, neuronal nitric oxide synthases; 3-AB, 3-Aminobenzamide; TBARS, thiobarbituric acid reactive substances; ANOVA, analysis of variance.

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Abstract

Methamphetamine (METH) administration in mice, results in a chronic dopamine (DA) depletion associated with nerve terminal damage, with DA oxidation and generation of reactive oxygen species (ROS) primarily mediating this neurotoxicity. The oxidative stress induced by METH putatively activates nuclear enzyme poly (ADP-ribose) polymerase (PARP), with excessive PARP activation eventually leading to cell death. In this study, we show that prevention of PARP activation by treatment with FR261529 (2-(4-chlorophenyl)-5-quinoxaline carboxamide), the compound which was recently identified as a novel PARP inhibitor (IC_{50} for PARP-1= 33 nM, IC_{50} for PARP-2= 7 nM), protects against both ROS-induced cells injury *in vitro* and METH-induced dopaminergic neuronal damage in an *in vivo* Parkinson's disease (PD) model. In PC12 cells, exposure of hydrogen peroxide or METH markedly induced PARP activation, and treatment with FR261529 (1μ M) significantly reduced PARP activation and attenuated cell death. In the mice METH model, METH (15mg/kg x 2, i.p., 2 hr apart) intoxication accelerated DA metabolism and oxidation in the striatum, with subsequent cell damage in nigro-striatal dopaminergic neurons after 4days. Oral administration of FR261529 (10 or 32mg/kg) attenuated the damage of dopaminergic neurons via marked reduction of PARP activity, and not via changes in dopamine metabolism or body temperature. These findings indicate that the neuroprotective effects of a novel PARP inhibitor, FR261529, were accompanied by inhibition of METH-induced PARP activation, suggesting that METH induces nigro-striatal dopaminergic neurodegeneration involving PARP activation and also orally active and brain penetrable PARP inhibitor FR261529 could be a novel attractive therapeutic candidate for neurodegenerative disorders such as PD.

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

Methamphetamine (METH) intoxication in mice produces neurodegeneration of dopaminergic terminals in the striatum and cell body loss in the substantia nigra pars compacta (SNpc) (Sonsalla et al., 1996; Hirata and Cadet, 1997; Kita et al., 1998). METH enters nerve terminals via the dopamine transporter (DAT) and displaces both vesicular and intracellular dopamine (DA). This displacement results in increased DA release within the cytoplasm of DA terminals, with subsequent DA oxidation by monoamine oxidase (MAO) activity and auto-oxidation to reactive oxygen species (ROS) and H_2O_2 (Cubells et al., 1994; Cadet and Brannock, 1998). The role of oxidative stress in the neurochemical actions of METH is supported by the fact that

METH can cause lipid and protein oxidation in mouse brain (Jayanthi et al., 1998; Gluck et al., 2001) and the administration of anti-oxidants, such as ascorbate and vitamin E, can attenuate METH-induced toxicity (De Vito and Wagner, 1989). Transgenic mice over-expressing the antioxidant enzyme copper/zinc superoxide dismutase showed an attenuated response to neurotoxic doses of METH (Cadet et al., 1994).

METH administration can also lead to increased levels of extracellular glutamate (Nash and Yamamoto, 1992), leading to excessive N-methyl-D-aspartate (NMDA) receptor activation and formation of superoxide and nitric oxide (Lafon-Cazal et al., 1993; Gunasekar et al., 1995). Superoxide reacts with nitric oxide to produce peroxynitrite, a highly cytotoxic compound, which causes neuronal death in numerous models of brain damage including cerebral ischemia and PD (Tabner et al., 2001). Pharmacological inhibition of various neuronal nitric oxide synthases (nNOS) reduces METH-induced neurotoxicity, and mice lacking the nNOS gene are resistant to this neurotoxicity (Di Monte et al., 1996; Itzhak et al., 1998).

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

Direct evidence for the involvement of PARP in the pathogenesis of METH-induced neurotoxicity comes from the evaluation of PARP inhibitors in mice.

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

We have recently identified FR261529 (2-(4-chlorophenyl)-5-quinoxaline carboxamide; chemical structure shown in Fig. 1) as a novel and potent PARP inhibitor, by applying the rational discovery strategies, such as structure-based drug design, combinatorial chemistry, and conventional SAR for improvement of potency. The purpose of the present study was, firstly, to investigate the PARP inhibiting activity and specificity of FR261529. The second purpose was to determine the PARP inhibitory properties and the neuroprotective properties of FR261529 in two *in vitro* experimental neuronal cell death models, in which PARP is markedly activated by H_2O_2 or METH exposure. Finally, the neuroprotective properties of FR261529 and the effect of FR261529 on DA release, lipid peroxidation and hyperthermia were evaluated in a mouse METH model, a relevant *in vivo* model of PD. 3-Aminobenzamide (3-AB) was also evaluated in this study as a reference PARP inhibitor.

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Materials and methods

Materials

Rat pheochromocytoma PC12 cells were purchased from American Type Culture Collection (Manassas, VA). FR261529, 2-(4-chlorophenyl)-5- quinoxaline carboxamide; (chemical structure shown in Fig.1) was synthesized at Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). 3-AB was purchased from Sigama-Aldrich (St.Louis, MO). METH (methamphetamine hydrochloride) was purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Tissue culture medium and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO) and tissue culture dishes were from Sumitomo (Osaka, Japan). Hydrogen peroxide (30%) and MTT [3-(4, 5-demethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide] were 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 materials 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 FR261529 and 3-aminobenzamide (3-AB), 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 testes 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 assay and various concentrations of FR261529 or 3-AB. The reaction

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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 was transferred onto GF/B filter (Packard Unifilter-GF/B) and washed three times with 10% TCA solution and 70% ethanol. After the filter was dried, the radioactivity was determined by liquid scintillation counting.

Preparation of nuclear extracts from PC12 cells and the mouse brain

Published methods were used for preparation of nuclear extracts, with minor modifications (Lahiri DK and Ge Y-W, 2000). To prepare the nuclear extracts from PC12 cells, 2 x 10^6 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 seconds 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 and placed 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 assay immediately.

For preparation of nuclear extracts from mouse brain, normal and/or drug treated whole brains were dissected and transferred to a Teflon homogenizer. Buffer A was added at 300 mg brain tissue per 1mL and ten strokes of homogenization were performed. The whole suspension was transferred equally to Eppendorf tubes followed by centrifugation in a microcentrifuge for 1 min. The supernatant containing

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mostly cytoplasmic constituents were removed, and 300μL 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 enzyme and DNA was used for the PARP assay immediately.

Specificity of FR261529

DAT binding assay: To determine the affinity of FR261529 and 3-AB to the dopamine transporter (DAT) and also to evaluate the level of cell damage after METH intoxication in mice, the striatum and/or substantia nigra were dissected from mice brain. Homogenate with KRH buffer containing 125mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 1.3mM $CaCl_2$, 1.2mM KH_2PO_4, 5.6mM glucose, 0.01mM nialamide, 25mM HEPES pH 7.4 was prepared by several centrifugations. For the DAT binding assay, mazindol binding to the DAT was evaluated according to the method of Javitch et al. (1984) with minor modifications. Binding was assayed by addition of membrane preparations from the 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 of ice-cold KRH buffer. The reaction solution was rapidly filtered through Whatman GF/C filters and three washes with binding buffer using a cell harvester. Nonspecific binding was determined in the presence of 10μM of cocaine.

Determination of [^{3}H]-spiperone binding activity: The affinity of FR261529 and 3-AB to dopamine D2 receptor was measured by using a

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[^{3}H]-spiperone binding assay as described previously (Zahnister and Dubocovich, 1983) with minor modifications. Briefly, the mice striatal membrane isolates (final 1mg/ml) were incubated with 50mM Tris-HCl buffer (pH 7.4, containing 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and 1mM EDTA) ,different concentration of drug and 3nM [^{3}H]-spiperone (specific activity 107Ci/mmol, Amersham) for 45min at 25°C, and processed as described above. Non-specific binding was determined in the presence of 10μM sulpiride.

Determination of MAO inhibitory activity: MAO 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 100μl of 67mM sodium phosphate buffer, pH 7.4 was pre-incubated in 96 well plates at 37°C for 10min. Following the pre-incubation, [^{14}C] 2-β-ethyl-1-phenylethylamine and different concentrations of FR261529 or 3-AB were 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 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.

Determination of radical scavenging activity and lipid peroxidation: To determine the radical scavenging activity of FR261529 and 3-AB, and also for measurement of lipid peroxidation in the striatum after dosing of METH in mice, TBARS (thiobarbituric acid reactive substances) were used with the modified method of Buege and Aust (1978) and Callaway et al. (1998). Briefly, mice brain

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synaptosomes were prepared from C57/BL6 mice (from Japan SLC, Shizuoka, Japan). To evaluate the inhibitory activity of FR261529 and 3-AB, different concentrations of each compound 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). 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 FR261529 or 3-AB 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

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[^{3}H]-citrulline production occurred in the absence of calcium.

Neuroprotective efficacy in PC12 cells

Determination of PARP activation: To determine PARP activation directly in PC12 cells, PARP enzyme assay and NAD assay were used. PC12 cells were seeded at $2x10^6$ cells in a F75 culture flask and cultured for 24hr. Then FR261529 was added to culture media at several concentrations. Thirty minutes later, cells were exposed H_2O_2 (0.1 to 1mM) or METH (0.1 to 1mM) for 30min and cells were detached using cell scraper and then nuclear extracts were prepared as described above. PARP activation was determined by PARP enzyme assay using nuclear extracts as a mixture of PARP enzyme and nicked DNA. To determine NAD level, detached cells were collected in microcentrifuge tube by centrifugation for 5 min x 100 g at 4°C. Cell homogenate was 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.

Assessment of cytotoxicity : PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) 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 experiments, 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 (0.1mM) and FR261529 or 3-AB for 6hr. To evaluate cell survival, MTT assay was performed according to a minor modification of the standard method. Briefly, MTT was added to the cultures at a final concentration of 0.2 mg/ml and after incubation at 37°C for 2 hr,

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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). In addition, hydrogen peroxide-induced cytotoxity was quantified by a standard measurement of lactate dehydrogenase (LDH) release with the use of the LDH assay kit (Wako, Japan).

Drug treatment: FR261529 was dissolved in 100% DMSO at 10^{-2} M and 3-AB was dissolved in PBS at 100mM, then both were diluted in DMEM without serum. These solutions were added to culture plate 0.5hr before H_2O_2 exposure.

METH-induced Parkinson's model in mice

Animals: For METH model, 9 to 10 week old male C57/BL6 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. On the day of the experiment, mice were maintained in a room temperature of 23°C. Rectal temperatures were recorded immediately before the first administration of drug and every hour thereafter using a small-animal rectal probe (TERUMO-CTM-303, Japan) in all experiments. 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 METH and PARP inhibitors: To determine the

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

Preparation of SN and striatum homogenate: For PARP assay and DAT binding assay using tissue homogenates, whole brains were dissected and put on ice-cold brain slicer immediately. One mm thick slice including SNpc and whole bilateral striata area was sectioned precisely by referring 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. The remaining tissues were used for preparation of the homogenate.

PARP inhibition in mouse brain: To estimate the PARP inhibition by treatment of FR261529 in mice brain, FR261529 was administered in normal C57BL/6 mice at 3.2 to 32mg/kg p.o. once, and each brain was dissected at two time points (1 and 4hr) after a single administration of FR261529. The brain homogenates were prepared immediately and PARP assay was conducted as described above.

Measurement of striatal DA, DOPAC, HVA and 3-MT levels: The contents of DA, DOPAC, HVA and 3-MT in the striatum were quantified using HPLC with electrochemical detection. Four days after the 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

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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 revesed phase catecholeamine column (SC-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. DA and its metabolites were quantified by peak height comparisons with standards run on the day of analysis. To determine whether FR261529 and other drugs affect dopamine or its metabolites content, a single dose of FR261529 (32mg/kg, p.o.), nomifensine (3.2mg/kg, i.p.), haloperidol (3.2mg/kg, i.p.) or apomorphine(3.2mg/kg, i.p.) was administered and the striatum was dissected after 1hr. The contents of DA, DOPAC, HVA and 3-MT in the striatum were quantified as described above.

Pharmacokinetic study in mice

Measurement of the concentration of FR261529 in plasma and brain were performed in mice following p.o. administration at 32 mg/kg. FR261529 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 the dosing, and the plasma and brain level of FR261529 were measured using high-performance liquid chromatography as described by Iwashita et al. (2004a).

Statistical analysis

The IC_{50} values obtained from studies in vitro and in vivo 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. Comparison between two groups was conducted using two-tailed Student's *t* -test. P-values less than 0.05 were considered to be significant.

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Results

PARP inhibitory activity of FR261529

To determine the species differences of PARP inhibitory activity of FR261529 and 3-AB, human recombinant PARP and nuclear extract from mouse brain were used. FR261529 potently inhibited the enzyme activity with an IC_{50} of 33 ± 0.9 nM and 35 ± 1.1 nM in human and mouse PARP, respectively (Table 1). The inhibitory activity of FR261529 was about 400-fold more potent than that of 3-AB (IC_{50} values of 3-AB in this study were 11200 ± 810nM and 11800 ± 690nM in human and mouse PARP, respectively). To confirm the selectivity of FR261529 and 3-AB, PARP-2 enzyme assay was also conducted. In our assay system, FR261529 was shown to be about 5-fold more potent for PARP-2 (IC_{50} of 7nM) than PARP-1 (IC_{50} of 33nM), compared with conventional non-selective PARP inhibitor 3-AB (IC_{50} for PARP-1 = 11.2μM and for PARP-2 =9.8μM).

Specificity of FR261529

A number of drugs which affect DA metabolism, the dopamine receptor, and dopamine transporter or generation of ROS or NO can prevent METH neurotoxicity. Therefore, to exclude the possibility that FR261529 could inhibit METH neurotoxicity directly, MAO-B inhibitory activity, DAT binding affinity and D2 receptor binding affinity were evaluated. In the MAO-B assay, FR261529 (IC_{50}: >10000nM) did not affect deamination of the substrate PEA in mouse brain homogenate, even with pre-incubation. Pargyline, which was used as a positive control, inhibited MAO-B activity with an IC_{50} value of 146nM. To determine the affinity of FR261529 to the dopamine transporter, a DAT binding assay was conducted using [^{3}H]-mazindol as a

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radioligand. In this assay, FR261529 did not inhibit mazindol binding to DAT even at a concentration of 10^{-5}M (IC_{50}: >10000nM), although nomifensine inhibited binding with an IC_{50} value of 201nM. While apomorphine, a D2 agonist, inhibited spiperone binding with an IC_{50} value of 201nM, FR261529 showed no inhibitory activity even at 10^{-5}M (IC_{50}: >10000nM). Radical scavenging activity and NOS inhibitory activity was evaluated using a TBARS assay and NOS catalytic activity assay, respectively. In TBARS assay, FR261529 did not inhibit TBARS production up to 10^{-5}M (IC_{50}: >10000nM), although vitamin E showed radical scavenging activity with an IC_{50} value of 228nM. In the NOS assay, 7-nitroindazole, a selective nNOS inhibitor, prevented NOS catalytic activity assessed by [^{3}H]-citrulline production with an IC_{50} value of 319nM, although FR261529 had no inhibitory activity up to 10^{-5}M (IC_{50}: >10000nM). 3-AB also showed very low affinity to DAT and D2 receptor, and no inhibitory activity to MAO and NOS. Radical scavenging activity of 3-AB was not observed in the TBARS assay (Table 1).

Neuroprotective action in PC12 cells

In this study, we first confirmed whether H_2O_2 or METH treatment induced PARP activation and cell death in PC12 cells. PARP activation was confirmed by measurement of the amount of incorporation of ^{32}P-NAD polymer in PC12 cells. Exposure of 0.1 to 1mM H_2O_2 for 30 min markedly increased incorporation of ^{32}P-NAD polymer and treatment with FR261529 inhibited the PARP activation from a concentration of 10^{-6}M (Fig. 2A). METH treatment similarly induced formation of ^{32}P-NAD polymer in PC12 cells (Fig. 2B). Treatment with FR261529 at concentrations higher than 10^{-6}M significantly inhibited METH-induced PARP activation. PARP activation by H_2O_2 exposure was also observed when assessed by NAD assay, and

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excessive PARP activation resulted in marked NAD depletion, and this NAD depletion was significantly inhibited by FR261529 treatment (Fig. 2C). Exposure of 0.1mM H_2O_2 for 6hr induced severe cell damage, although FR261529 treatment at a concentration range from 10^{-6}M significantly attenuated cell death as evaluated by MTT assay (Fig.3A) and LDH assay (Fig.3B). A similar neuroprotective effect was observed in PC12 cells treated with 3-AB at concentrations higher than 10^{-4}M (Fig. 3C&D).

Pharmacokinetic study in mice

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

Neuroprotective action in mouse METH model

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

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

Brain PARP inhibition by treatment with FR261529: To explore the optimal dosing of FR261529 in mouse METH model, PARP inhibition by FR261529 treatment in mouse brain was determined by using PARP enzyme assay. FR261529 (3.2, 10 or 32 mg/kg) was orally administered in normal mice and nuclear extracts were prepared from mouse brain at 1hr and 4hr after dosing. All doses of FR261529 significantly inhibited PAR polymer formation induced by addition of nicked DNA in the PARP enzyme assay at 1hr after dosing. Higher doses of FR261529 (10 and 32 mg/kg) moderately but still significantly inhibited PAR polymer formation at 4 hr (Fig. 4).

Neuroprotective effects of FR261529 and 3-AB: FR261529 was administered to mice at doses of 3.2, 10 or 32 mg/kg p.o. twice (1h prior to first and 1hr after second injection of METH), and the striatum was dissected four days later to quantify DA and its metabolites. In this paradigm, treatment of FR261529 prevented the depletion of striatal DA, DOPAC and HVA content in a dose-dependent manner,

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and with statistically significant protection at higher doses (10 and 32mg/kg; Fig. 5A). 3-AB similarly significantly attenuated the depletion of DA and its metabolites contents at higher doses (320 mg/kg) (Fig. 5B). Furthermore, treatment of FR261529 ameliorated the damage of the nigrostriatal dopaminergic terminals in the striatum, as assessed by DAT binding (Fig. 5C). The depletion of DAT binding sites following METH intoxication and its % recovery by FR261529 treatment was consistent with the degree of DA depletion and its recovery, respectively.

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

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

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Effect on DA metabolism in the striatum: The efficacy of several drugs with protective effects against METH toxicity was analyzed by measuring DA and its metabolite contents 1hr after METH injection. Consistent with previous reports (Cosi et al., 1996; Fornai et al., 2001), administration of a DAT inhibitor (nomifensine: 3.2mg/kg, i.p.), a D2 receptor agonist (apomorphine: 3.2 mg/kg, i.p.) or a D2 receptor antagonist (haloperidol: 3.2 mg/kg, i.p.) affected the DA and its metabolite contents at 1hr after dosing (Table 3A). In contrast, FR261529 administration did not modify striatal DA, DOPAC nor HVA levels (Table 3B).

Effects on body temperature: Rectal temperature was significantly elevated within 1hr after the first injection of METH (2x15mg/kg, 2hr apart) as compared with the saline treated control mice. This hyperthermia by METH was maintained until 3hr after the second injection of METH, and all doses of FR261529 (3.2, 10 and 32 mg/kg; twice, 4hr apart) did not induce a significant hypothermic effect (Fig.8).

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Discussion

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

METH enters DA terminals via the dopamine transporter (DAT), and displaces both vesicular and intracellular DA. This results in increased DA release within the cytoplasm of DA terminals, where DA is oxidized by MAO activity and auto-oxidation to ROS and hydrogen peroxide (Cubells et al., 1994; Cadet and Brannock, 1998). METH administration can also lead to increased extracellular glutamate (Nash and Yamamoto, 1992), which, in turn, induces NMDA receptor activation, linking to the formation of superoxide and nitric oxide (Lafon-Cazal et al.,

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1993; Gunasekar et al., 1995; Yamamoto and Zhu, 1998). Superoxide may react with nitric oxide produced by NOS to form the potent oxidant peroxynitrite, which damages intracellular proteins and DNA to cause cell death. Although PARP is activated following DNA damage that mediates neuronal cell death, providing a mechanism by which PARP inhibitor such as benzamide can exert neuroprotection against METH toxicity (Cosi et al., 1996), drugs that affect DA release and metabolism can also block METH-induced cell death. To differentiate between these mechanisms, we determined if neuroprotection of FR261529 is achieved by inhibition of binding to DA receptor, binding to DAT and MAO activity. FR261529 has no inhibitory potential against these activities, suggesting that FR261529 is not able to affect DA uptake or inhibit accelerated DA metabolism.

The role of NOS in METH-induced dopaminergic neurotoxicity, neuroprotection by various nNOS inhibitors, along with the evidence that lacking the nNOS gene in mice are resistant to METH-induced dopaminergic neurotoxicity have been reported (Di Monte et al., 1996; Itzhak et al., 1998). Furthermore, the administration of anti-oxidants can attenuates METH-induced neurotoxicity (De Vito and Wagner, 1989) and transgenic mice over-expressing the anti-oxidant enzyme copper/zinc SOD showed an attenuated response to neurotoxic doses of METH have been reported (Cadet et al., 1994). These results indicate that radical scavengers and nNOS inhibitors can block METH-induced dopaminergic neuron damage. FR261529 had no antioxidant properties or nNOS inhibitory activity even at a concentration of 10^{-5}M, suggesting that neither radical scavenging activity nor nNOS inhibition is involved in the action of FR261529. While the effects of FR261529 on other diverse classes of known neurotransmitter receptors, ion channels or enzymes have not been fully clarified, the neuroprotective properties are likely the consequence of its specific

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PARP inhibitory activity.

To determine PARP inhibitory and neuroprotective properties of FR261529 in cultured cells, PARP activation and cell damage were induced by H_2O_2 or METH exposure in PC12 cells. In this cell death system, H_2O_2 exposure markedly induced PARP activation, concomitant NAD depletion, and early-stage cell death. Inconsistent with the potent PARP inhibitory activity of FR261529 (IC_{50} = 33nM), this compound attenuated both PARP activation and cell death at a concentration higher than 1000nM (30-fold difference), suggesting that FR261529 does not have good cell permeability compared with that of 3-AB, another widely used PARP inhibitor (IC_{50} =11.2μM versus MEC=100μM; <10-fold difference) and quinoxaline derivatives (<10-fold difference; data not shown). Other quinoxaline derivatives of FR261529 also showed a discrepancy between PARP inhibitory activity in enzyme assay and neuroprotective properties in cultured cells (data not shown). Therefore, we speculate that the relatively low potency of neuroprotective properties of FR261529 and its derivatives in mouse METH model results from, in part, its poor permeability across the cell membrane.

METH-induced dopaminergic neuronal damage, assessed by DA and metabolites contents, was well correlated with the reduction of DAT binding sites in the striatum. Although the reduction of DAT binding sites in the SNpc was relatively small compared with that in the striatum, a good correlation was also observed between the depletion of DAT binding sites in the striatum and SNpc. In our preliminary study, we confirmed that the reduction of TH-positive neurons in the SNpc correlated with the decrease in DA contents in the striatum. These results support the notion that the depletion of DAT binding sites is consistent with damage in the striatum and the loss of cell bodies in the SNpc, and that the DAT binding assay is a

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suitable substitute for quantifying the dopaminergic cell death with TH immunonostaining.

The most important finding in the present study was that the treatment of FR261529 following METH intoxication dose-dependently ameliorated the depletion of DA and its metabolites, as well as that of DAT binding sites in the striatum. FR261529 was orally active, with a brain concentration of FR261529 (32 mg/kg, p.o.) of 1.49 μg/g, which is estimated as higher than 10^{-6}M, at 2hr post-dosing. Thus, this dosing regimen yielded sufficient brain levels of FR261529 to exert PARP inhibitory activity. Although FR261529 attenuated the depletion of DAT binding sites in the SNpc, the amelioration was not statistically significant because of the small reduction by METH treatment (DAT binding site in the SNpc was 85.8% for vehicle control and 91.1% for FR261529 treatment compared with Normal; data not shown). Consistent with these data, METH-induced PARP activation in the SNpc was undetectable following two dosings of METH. However, four doses of METH induced PARP activation at a detectable level, and the neuroprotective dose of FR261529 significantly inhibited PARP activation, supporting our view that METH neurotoxicity leads to PARP activation, and that the neuroprotective properties of FR261529 involve specific PARP inhibitory activity.

The role of oxidative stress in the biochemical actions of METH is supported by the fact that METH can cause lipid and protein oxidation in brain (Jayanthi et al., 1998; Gluck et al., 2001) and the administration of anti-oxidants attenuates METH-induced toxicity (De Vito and Wagner, 1989). Furthermore, drugs which inhibit lipid peroxidation are neuroroprotective against METH neurotoxicity in the striatum (Cadet et al., 1994). In the present study, METH-induced lipid peroxidation in the striatum was observed at 2hr after METH treatment, and drugs that are

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neuroprotective against METH neurotoxicity, such as the DAT inhibitor nomifensine and the D2 receptor antagonist haloperidol, effectively attenuated the production of malondialdehyde (MDA). However, the PARP inhibitors 3-AB and FR261529, at neuroprotective doses, had no influence on MDA generation following METH treatment. These results offer supportive evidence that METH produces oxidative damage in vivo, but that FR261529 does not inhibit the generation of METH-induced radical formation.

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

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

neurodegenerative pathways, but also an attractive therapeutic candidate for neurodegenerative disorders such as Parkinson's disease.

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References

Abdelkarim GE, Gertz K, Harms C, Katchanov J, Dirnagl U, Szabo C and Endres M (2001) Protective effects of PJ34, a novel, potent inhibitor of poly (ADP-ribose) polymerase (PARP) in in vitro and in vivo models of stroke. *Int. J. Mol. Med.* 7(3): 255-260

Albers DS and Sonsalla PK (1995) Methamphetamine-induced hyperthermia and dopaminergic neurotoxicity in mice: pharmacological profile of protective and nonprotective agents. *J. Pharmacol. Exp. Ther.* 275: 1104-1114

Ali SF, Newport GD, Holson RR, Slikker W Jr. and Bowyer JF (1994) Low environmental temperatures or pharmacologic agents that produce hypothermia decrease methamphetamine neurotoxicity in mice. *Brain Res.* 658: 33-38

Banasik M, Komura H, Shimoyama M, and Ueda K (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl) transferase. *J Biol Chem* 267:1569-1575

Bowyer JF, Tank AW, Newport GD, Slikker W Jr., Ali SF and Holson RR (1992) The influence of invironmental temperature on the transient effects of methamphetamine on dopamine levels and dopamine release in the rat striatum. *J. Pharmacol. Exp. Thr.* 260: 817- 824

Buege JA and Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol.* 52: 302-310

Callaway JK, Beart PM and Jarrott B (1998) A reliable procedure for comparison of antioxidants in rat brain homogenates. *J. Pharmacol. Toxicol. Methods.* 39 (3): 155-162

Cadet JL, Sheng P, Ali S, Rothman R, Carlson E and Epstein C (1994) Attenuation of

methamphetamine-induced neurotoxicity in copper/zinc superoxide dismutase transgenic mice. *J. Neurochem.* 62: 380-383

Cadet JL and Brannock C (1998) Free radicals and the pathobiology of brain dopamine systems. *Neurochem. Int.* 32: 117-131

Cosi C, Chopin P and Marien M (1996) Benzamide, an inhibitor of poly (ADP-ribose) polymerase, attenuates methamphetamine-induced dopamine neurotoxicity in the C57BL/6N mouse. *Brain Res.* 735(2): 343-348

Cubells JF, Rayport S, Rajendran G and Sulzer D (1994) Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. *J. Neurosci.* 14: 2260-2271

de Murcia G, Schreiber V, Molinate M, Saulier B, Poch O, Masson M, Niedergang C and Menissier J (1994) Structure and function of Poly(ADP-ribose) polymerase. *Mol. Cell Biochem.* 138: 15-24

De Vito MJ and Wagner GC (1989) Methamphetamine-induced neuronal damage: a possible role for free radicals. *Neuropharmacology* 28: 1145-1150

Di Monte DA, Royland JE, Jakowec MW and Langston JW (1996) Role of nitric oxide in methamphetamine-induced neurotoxicity: protection by 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase. *J. Neurochem.* 67: 2443-2450

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH and Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* 3: 1089-1095

Fornai F, Battaglia G, Gesi M, Orzi F, Nicoletti F and Ruggieri S (2001) Dose-dependent protective effects of apomorphine against methamphetamine-induced nigrostriatal damage. *Brain Res.* 898(1): 27-35

Gluck MR, Moy LY, Jayatilleke E, Hogan KA, Mizuno L and Sonsalla PK (2001) Parallel increases in lipid and protein oxidative markers in several mouse brain regions after methamphetamine treatment. *J. Neurochem.* 79: 152-160

Gunasekar PG, Kanthasamy AG, Borowitz JL and Isom GE (1995) NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implications for cell death. *J. Neurochem.* 65: 2016-2021

Ha HC and Snyder SH (1999) Poly (ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc. Natl. Acad. Sci. USA* 96: 13978-13982

Hirata H and Cadet JL (1997) p53-knockout mice are protected against the long-term effects of methamphetamine on dopaminergic terminals and cell bodies. *J. Neurochem.* 69: 780-790

Huang PL, Dawson TM, Bredt DS, Snyder SH, and Fishman MC (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75:1273-1286

Itzhak Y, Gandia C, Huang PL and Ali SF (1998) Resistance of neuronal nitric oxide synthase-deficient mice to methamphetamine-induced dopaminergic neurotoxicity. *J. Pharmacol. Exp. Ther.* 284: 1040-1047

Iwashita A, Yamazaki S, Mihara K, Hattori K, Yamamoto H, Ishida J, Matsuoka N and Mutoh S (2004a) Neuroprotective effects of 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. *J. Pharmacol. Exp. Ther.* : In press

Iwashita A, Tojo N, Matsuura S, Yamazaki S, Kamijo K, Ishida J, Yamamoto H, Hattori K, Matsuoka N and Mutoh S (2004b) A novel and potent PARP-1 inhibitor, 5-chloro-2-[3-(4-phenyl-3, 6-dihydro-1(2H)-pyridinyl)propyl]-4(3H)-quinazolinone

(FR247304), attenuates neuronal damage in vitro and in vivo models of cerebral ischemia. *J. Pharmacol. Exp. Ther.*: In press

Jayanthi S, Ladenheim B and Cadet JL (1998) Methamphetamine-induced changes in antioxidant enzymes and lipid peroxidation in copper/zinc-superoxide dismutase transgenic mice. *Ann. N.Y. Acad. Sci.* 844: 92-102

Javitch JA, Blaustein RO and Snyder SH (1984) [3H]mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. *Mol. Pharmacol.* 26: 35-44

Kita T, Paku S, Takahashi M, Kubo K, Wagner GC and Nakashima T (1998) Methamphetamine-induced neurotoxicity in BALB/c, DBA/2N and C57BL/6N mice. *Neuropharmacology* 37: 1177- 1184

Lahiri DK and Ge Y-W (2000) Electrophoretic mobility shift assay for the detection of specific DNA-protein complex in nuclear extracts from the cultured cells and frozen autopsy human brain tissue. *Brain Res. Protocols.* 5: 257-265

LaPlaca MC, Zhang J, Raghupathi R, Li JH, Smith F, Bareyre FM, Snyder SH, Graham DI and McIntosh TK (2001) Pharmacologic inhibition of poly (ADP-ribose) polymerase is neuroprotective following traumatic brain injury in rats. *J Neurotrauma* 18(4): 369-376

LaVoie MJ and Hastings TG (1999) Dopamine quinine formation and protein modification associated with the striatal neurotoxicity of methamphetamine: evidence against a role for extracellular dopamine. *J. Neurosci.* 19: 1484-1491

Nash JF and Yamamoto BK (1992) Methamphetamine neurotoxicity and striatal glutamate release: comparison to 3,4-methyl-enedioxymethamphetamine. *Brain Res.* 58: 237-243

Sonsalla PK, Jochnowitz ND, Oostveen JA and Hall ED (1996) Treatment of mice with methamphetamine produces cell loss in the substantia nigra. *Brain Res.* 738:

172-175

Szabo C, Zingarelli B, O'Connor M and Salzman AL (1996) DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle exposed to peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* 93: 1753-1758

Tabner BJ, Turnbull S, El-Agnaf O and Allsop D (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr. Top. Med. Chem.* 1: 507-517

Takahashi K, Pieper AA, Croul SE, Zhang J, Snyder SH and Greenberg JH (1999) Post-treatment with an inhibitor of poly (ADP-ribose) polymerase attenuates cerebral damage in focal ischemia. *Brain Res.* 829(1-2): 46-54.

Yamamoto BK and Zhu W (1998) The effects of methamphetamine on the production of free radicals and oxidative stress. J. Pharmacol. Exp. Ther. 287: 107-114

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM and Dawson VL (2002) Mediation of poly (ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science (Wash DC)* 297: 259-263

Zahniser NR and Dubocovich ML (1983) Comparison of dopamine receptor sites labeled by [^{3}H]-S-sulpiride and [^{3}H]-spiperone in striatum. *J. Pharmacol. Exp. Ther.* 227: 592-599

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Figure legends

Figure 1

Chemical structure of FR261529, 2-(4-chlorophenyl)-5- quinoxalinecarboxamide

Figure 2

Inhibition of hydrogen peroxide- or METH-induced PARP activation by FR261529 in PC12 cells. Exposure of 0.1 to 1mM H_2O_2 (A) or METH (B) for 30 min dose-dependently induced marked PARP activation, as evaluated by the incorporation of ^{32}P-NAD polymer (235 ± 7.8 cpm/10^6cells in the non-treated control group). FR261529 at concentrations above 10^{-6}M attenuated both H_2O_2 - and METH-induced PARP activation, and at a 10^{-5}M FR261529 prevented PARP activation completely (A and B). PARP activation was also confirmed by NAD depletion following H_2O_2 (100μM) exposure. FR261529, at concentrations above 10^{-6}M, attenuated H_2O_2 -induced NAD depletion (C). Each point represents the mean ± S.E.M. of at least three experiments. *, P<0.05, **, P<0.01 versus non-treated control group, and ##, P<0.01 versus vehicle treated control group (by one-way ANOVA followed by Dunnett's multiple comparison test). ###, P<0.001 versus H_2O_2 treated control group (by Student's t-test).

Figure 3

Neuroprotective effects of FR261529 (A and B) and 3-AB (C and D) against H_2O_2 induced cytotoxicity in PC12 cells. Exposure of 100μM H_2O_2 for 6hr produced severe cell damage, as evaluated by MTT assay (A and C) and LDH assay (B and D). Neuronal cell death was significantly reduced by pre-treatment (0.5hr prior to H_2O_2 exposure) of FR261529 (A and B) or 3-AB (C and D) at a concentration from 10^{-6}M or

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from 10^{-6}M, respectively. 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<0.001 versus H_2O_2 treated control group (by Student's t-test).

Figure 4

FR261529 (p.o.) inhibits PARP activation in normal mouse brain. Normal mice treated with 3.2, 10 or 32mg//kg of FR261529 were decapitated after 1and 4hr, and nuclear extracts containing PARP enzyme and drug were prepared for *in vitro* PARP assay to evaluate the PARP inhibition level by FR261529. At 1hr after treatment of FR261529, all doses of this compound significantly inhibited PARP activation induced by addition of nicked-DNA in an *in vitro* assay system. At 4hr, only the higher dose of FR261529 (10 and 32mg/kg) slightly but significantly inhibited PARP activation in this assay system. PARP activations represent the incorporation of ^{32}P-NAD polymer (8230 ± 339 cpm/10mg brain tissue in the non-treated control group). 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 5

Neuroprotective effects of PARP inhibitors FR261529 and 3-AB in the mouse METH model. For evaluation of FR261529 and 3-AB in mouse METH model, two dosings of METH (15mg/kg, 2hr apart) and drug (twice at 1h prior to first injection of METH and then 4hr later) paradigms were developed. The striatum was dissected from each mouse at 4 days after METH administration. In the non-treated control group, the contents of DA, DOPAC and HVA were 13.97 ± 0.18, 0.82 ± 0.03 and 1.41 ± 0.05

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ng/mg tissue, respectively. (A) Treatment of FR261529 dose-dependently attenuated the depletion of striatal DA, DOPAC and HVA contents, and its effect was statistically significant at higher doses (10 and 32mg/kg). (B) Administration of 3-AB partially prevented the depletion of DA, DOPAC and HVA contents in the striatum, and its effect was statistically significant (C) FR261529 treatment also attenuated the depletion of DAT binding sites in the striatum, and its effects were dose-dependent and significant at higher doses (10 and 32mg/kg). Data represent the % of the non-treated control group (683 ± 16.4 fmol/mg tissue). Values are means ± S.E.M of n=6-7 mice. Significant difference from control value are indicated by * for $P<0.05$, ** for $P<0.01$ versus vehicle treated control group (by one-way ANOVA followed by Dunnett's multiple comparison test), and by ## for $P<0.01$ versus vehicle treated control group (by Student's t-test).

Figure 6

Effects of FR261529 on METH-induced PARP activation in the SNpc. METH (15 mg/kg) was intraperitoneally injected twice at 2hr intervals and FR261529 (32mg/kg, p.o.) was administered twice by p.o. at 1hr prior to first injection and 1hr after fourth injection of METH. The SNpc was dissected from each mouse at 1hr of last administration of FR261529 and determined PARP activation used by PARP enzyme assay. Two dosing of METH slightly but significantly increased PARP activation, and treatment FR261529 prevented PARP activation completely. PARP activations represent the incorporation of ^{32}P-NAD polymer (297 ± 20.8 cpm/10mg brain tissue in the non-treated control group). Data are presented as mean ± S.E.M of 5-6 mice. Significant difference from control value are indicated by * for $P<0.05$ versus vehicle treated control group and by # for $P<0.05$ versus FR261529 treated group (by

Student's test).

Figure 7

METH-induced lipid peroxidation in the striatum. The degree of lipid peroxidation following METH treatment was measured using by TBARS assay. (A) METH treatment (2 x15mg/kg, 2hr apart) time-dependently increased the generation of MDA in the striatum specifically, and its effect persisted on until 24hr after last METH treatment. (B) Administration of nomifensine or haloperidol which has protective potential in mouse METH model significantly attenuated the MDA production at 2hr after METH treatment. However, neuroprotective dose of 3-AB or FR261529 did not show any change of MDA production. Data are presented as mean ± S.E.M of 5-6 mice. Significant difference from control value are indicated by ** $P<0.01$, statistically significant compared with control group (by Student's t-test).

Figure 8

Effect of FR261529 on body temperature during METH-treatment in mice. METH-induced hyperthermia was observed immediately after first injection of METH treatment (2 x 15mg/kg, 2hr apart), and persisted on 4hr later. Treatment of FR261529 at doses from 3.2 to 32mg/kg did not show hypothermic action and did not affect hyperthermia of METH treatment. Data are presented as mean ± S.E.M of 5-6 mice. Significant difference from normal group are indicated by ** $P<0.01$ (by Student's t-test).

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TABLE 1

Pharmacological characterization of 3-AB and FR261529

	IC_{50} (nM)						
	Human PARP inhibition	Mouse PARP inhibition	[^{3}H] spiperone binding	DAT binding	TBARS	MAO	NOS
FR261529	33 ± 0.9	35 ± 1.1	>10000	>10000	>10000	>10000	>10000
3-AB	11200 ± 810	11800 ± 690	>10000	>10000	>10000	>10000	>10000
reference	—	—	apomorphine 223 ± 8.7	nomifensine 201± 6.8	vitamin E 228± 8.1	pargyline 146± 5.1	7-NID 319± 5.7

Pharmacological characterization of 3-AB and FR261529. PARP inhibitory activity of FR261529 and 3-AB were evaluated in human recombinant PARP and mouse PARP from nuclear extracts of mouse brain. IC_{50} values were calculated from the concentration dependence of the inhibition curves by using computer-assisted non-linear regression analyses. To determine pharmacological potential of FR261529 and 3-AB, D2 receptor binding assay, DAT binding assay, MAO assay, TBARS assay and NOS assay were conducted. FR261529 and 3-AB did not exhibit D2 receptor affinities, DAT affinities, radical scavenging activity, MAO inhibitory activity and NOS inhibitory activity at a concentration from 10^{-8} to 10^{-5}M in all assays, and all IC_{50} values were under 10000nM. PARP inhibitory activities are means ± S.E.M. of n=3 determinations tested in triplicate.

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TABLE 2

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

Brain area	assay	METH dosing (i.p., 2hr interval)				
		saline	2 x 10 mg/kg	2 x 15 mg/kg	2 x 25 mg/kg	4 x 15 mg/kg
Striatum	DA content	100 ± 2.8	69.4 ± 4.6 **	42.1 ± 8.6 **	20.3 ± 4.6 **	9.4 ± 0.4 **
	DAT binding site	100 ± 4.5	70.8 ± 4.1 **	42.6 ± 5.2 **	24.8 ± 5.7 **	11.7 ± 0.9 **
SNpc	DAT binding site	100 ± 3.1	95.8 ± 7.7	85.8 ± 4.6 *	82.5 ± 7.3 *	66.1 ± 5.8 **

* P<0.05, ** P<0.01 vs control by Dunnett's test

Comparison with dopamine content and DAT biding site in striatum following METH intoxication in mice. METH (2 x 10, 2 x 15, 2 x 25 or 4 x 15mg/kg) was injected intraperitoneally at 2hr intervals and dopamine content and DAT binding site in the striatum and DAT binding site in the SNpc were measured after 4day. Both striatal dopamine content (14.21 ± 0.40 ng/mg tissue in the non-treated control group) and DAT binding site (640 ± 28.8 fmol/mg tissuue in the non-treated control group) were significantly and dose-dependently decreased by METH treatment after 4day. Decrease of dopamine content level was coincident with that of DAT binding site. DAT binding site in the SNpc (152 ± 4.7 fmol/mg tissue in the non-treated control group) was also dose-dependently decreased by METH treatment, and at higher doses the depletion was statistically significant. Values are means ± S.E.M. of n=5-6 mice. **P<0.01, statistically significant compared with control group (by Student's t-test).

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TABLE 3

A) Protective effect of several drugs and low temperature against METH neurotoxicity

	METH model (2 x 15mg/kg, i.p.)
	(% Recovery of DA content)
FR261529 (32mg/kg)	**48 ± 3.9 ****
Nomifensine (3.2mg/kg)	**42 ± 2.5 ****
Haloperidol (3.2mg/kg)	**103 ± 3.2 ****
Low temperature (18℃)	**67 ± 2.8 ****

B) Dopamine and its metabolites contents at 1hr following drug administration

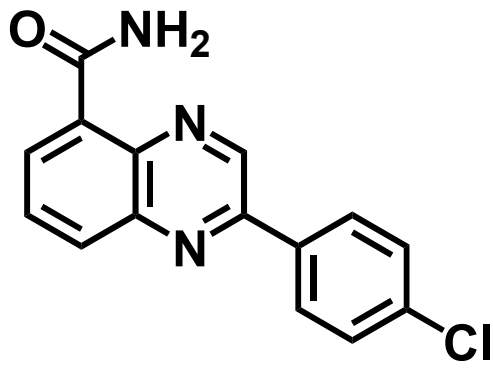
(Drug)	(mg/kg)	(route)	DA	DOPAC	HVA	3-MT
METH	**-**	**-**	**106.1 ± 5.6**	**42.8 ± 1.4 ****	**156.5 ± 9.1 ****	**361.5 ± 21.6 ****
FR261529	**32**	**p.o.**	**96.1 ± 3.9**	**95.8 ± 7.8**	**104.3 ± 3.8**	**98.8 ± 3.2**
Nomifensine	**3.2**	**i.p.**	**95.8 ± 4.2**	**54.2 ± 2.2 ****	**77.5 ± 2.2 ****	**94.2 ± 6.6**
Haloperidol	**3.2**	**i.p.**	**50.9 ± 2.1 ****	**386.3 ± 18.1 ****	**209.5 ± 9.4 ****	**53.6 ± 5.1 ****
Apomorphine	**3.2**	**i.p.**	**98.7 ± 1.8**	**62.9 ± 5.4 ****	**49.2 ± 4.6 ****	**67.8 ± 3.1 ****

** values different from the non-treatment control group (P<0.01)

Protective effect of several drugs against METH neurotoxicity (A) and dopamine and its metabolites contents at 1hr following drug administration (B). Administration of FR261529 (PARP inhibitor: 32mg/kg, p.o.), DAT inhibitor (nomifensine: 3.2mg/kg, i.p.), D2 receptor agonist (apomorphine: 3.2mg/kg, i.p.) and D2 receptor antagonist (haloperidol: 3.2mg/kg, i.p.) showed a protective potential against METH neurotoxicity at 4days following METH and drug administration in mouse METH model. % recovery of DA content was calculated as follows: % recovery of DA contents = {(A-B)/(C-B)} x 100, where A is the DA content in drug-treated mice, B is the DA contents in vehicle-treated mice and C is the DA contents in Normal mice (14.73 ± 0.31 ng/mg tissue) (A). These drugs affected the DA and its metabolite contents at 1hr after

dosing in Normal mice, however, FR261529 even at a neuroprotective dose in mouse METH model did not modify striatal DA and its metabolites DOPAC, HVA and 3-MT levels, compared with vehicle-treated mice. In the non-treated control group, the concentrations of DA and its metabolites DOPAC, HVA and 3-MT were 14.17 ± 0.39, 0.84 ± 0.02, 1.40 ± 0.03 and 0.25 ± 0.02, respectively (B). Data are presented as mean ± S.E.M. of 5-6 mice. Significant difference from control value are indicated by ** $P<0.01$ (by Student's t-test).

Figure 1



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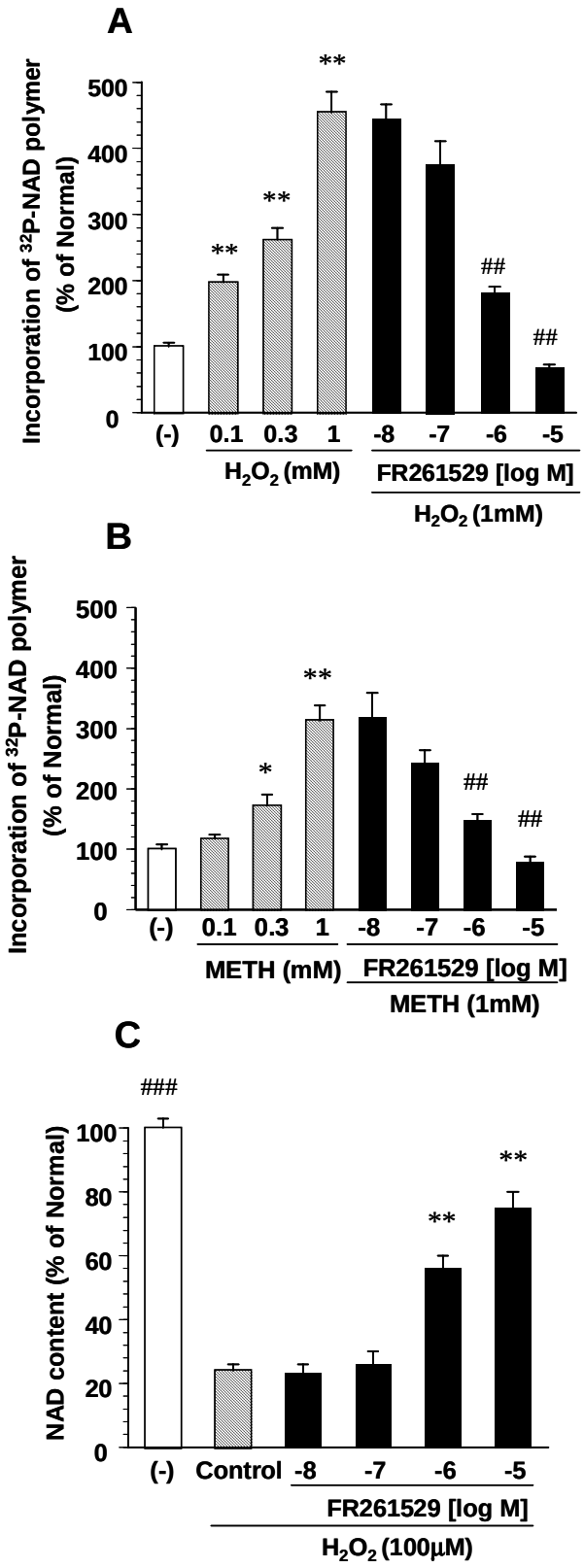

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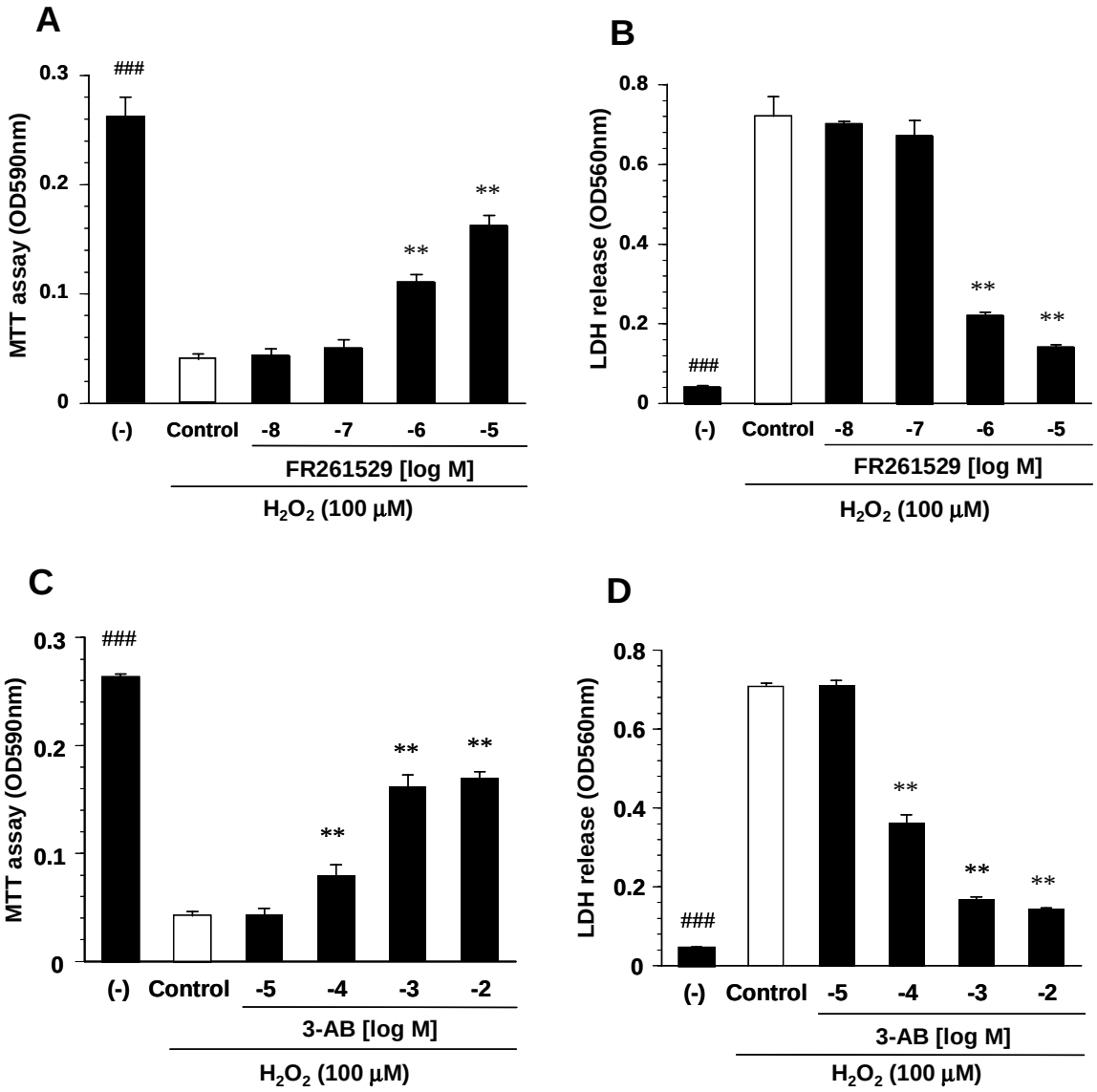

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Figure 4

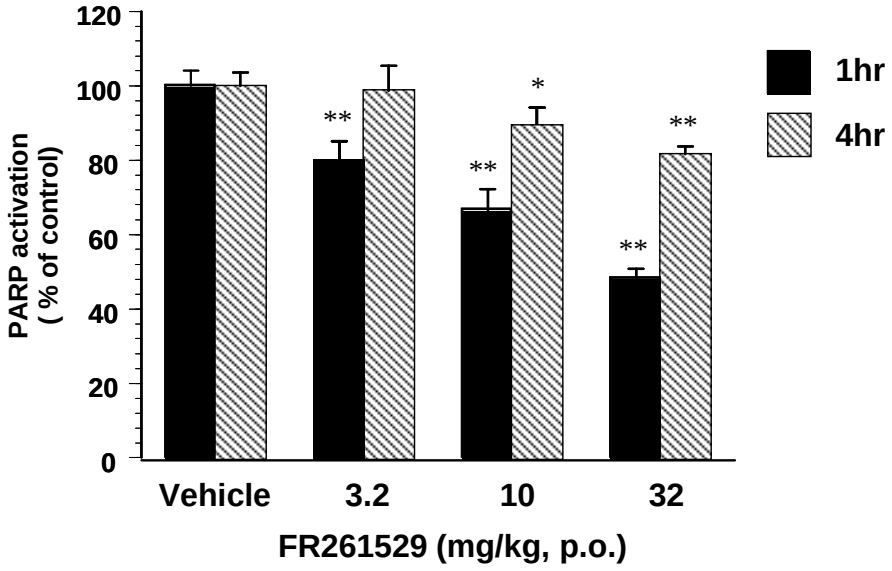

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Figure 5

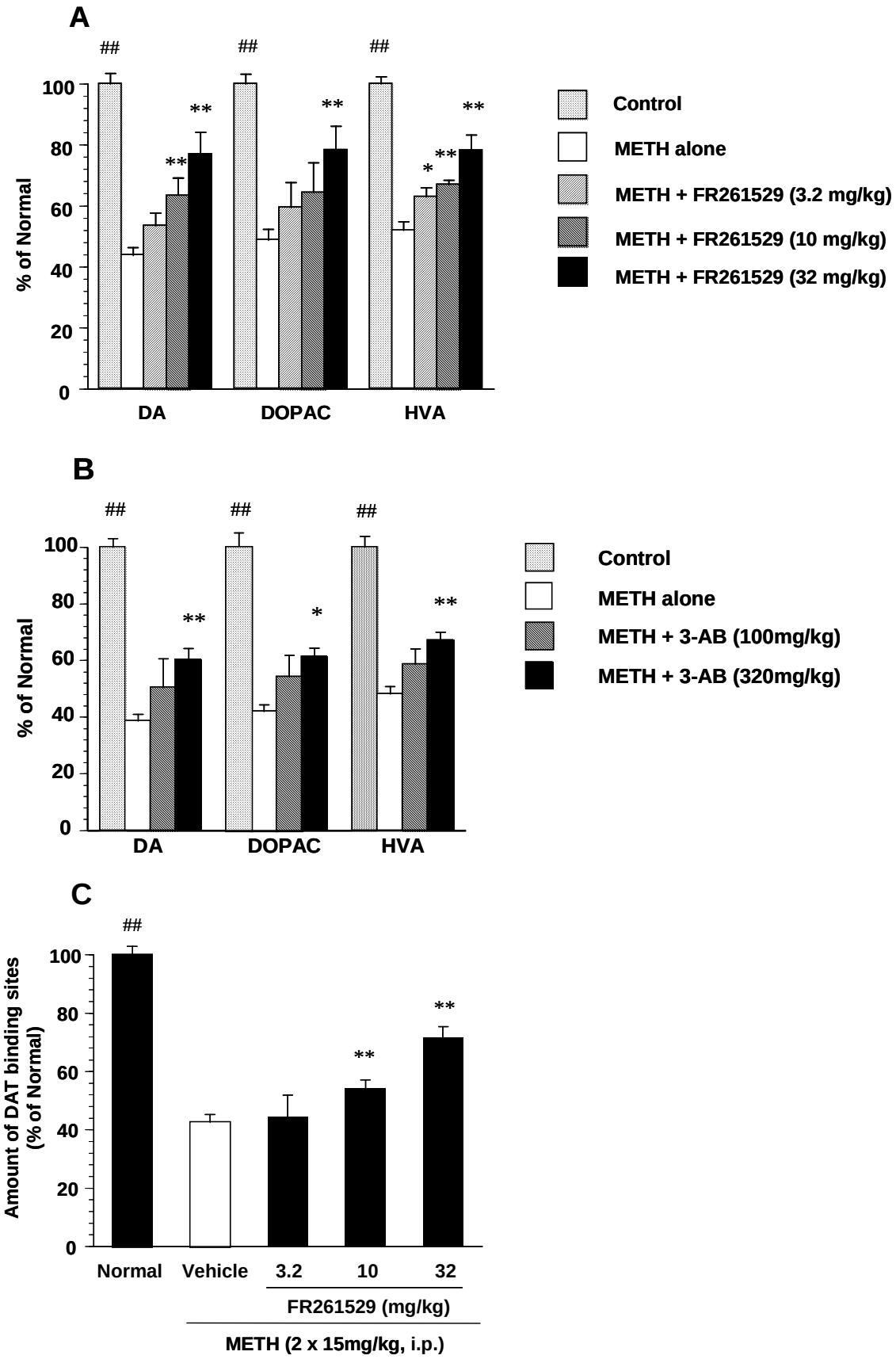

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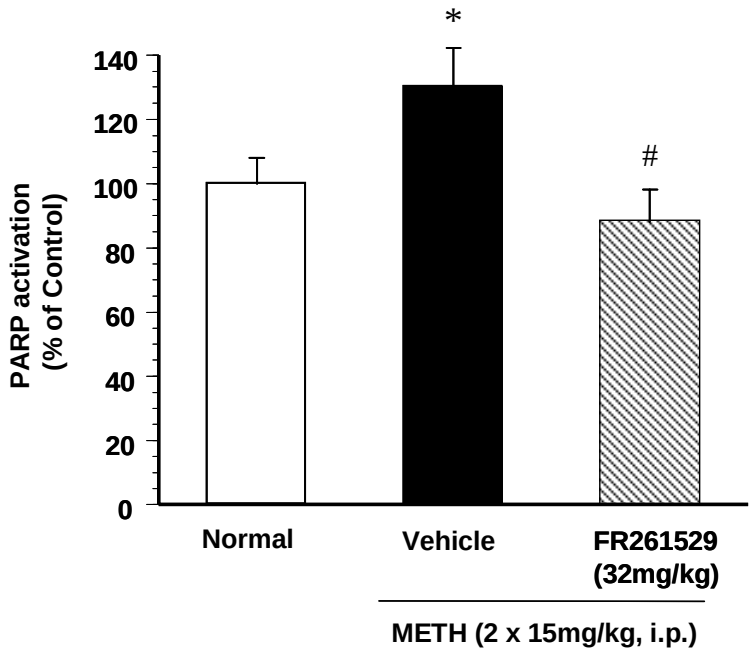
Figure 6



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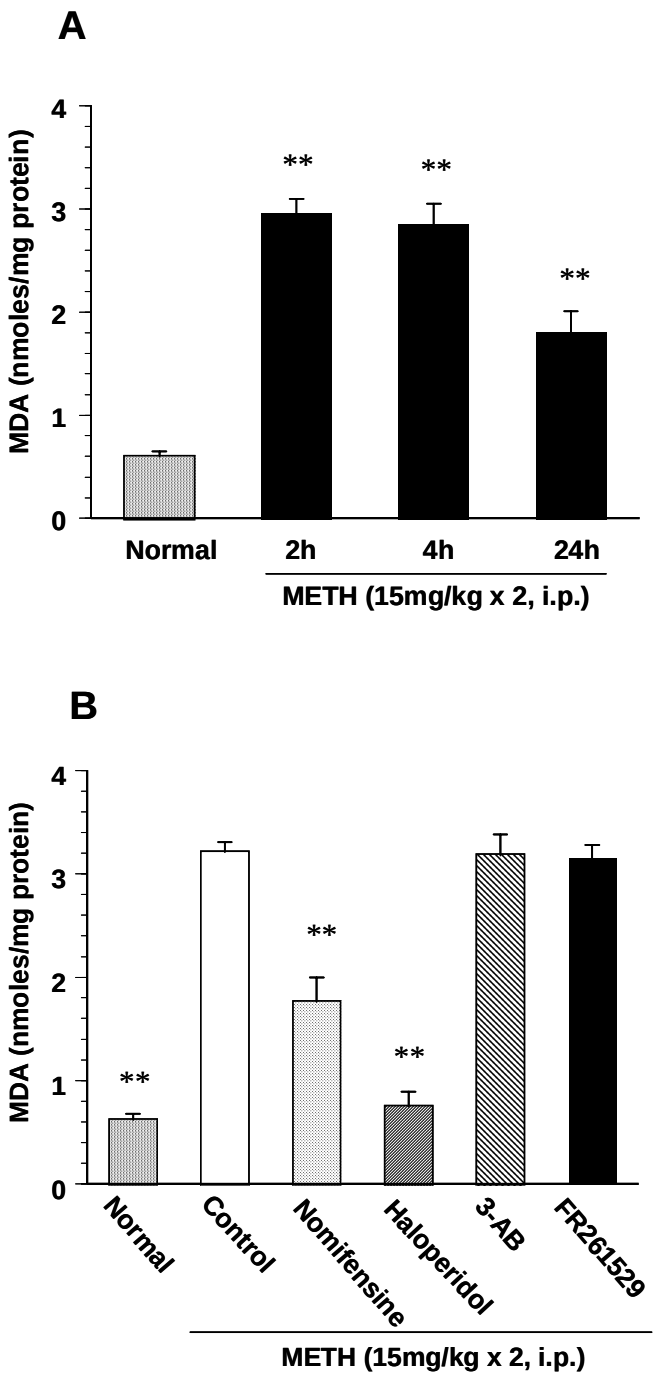

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Figure 8