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A chemical compound commonly used to inhibit PKR, {8-(imidazol-4-ylmethylene)-6H-azolidino[5,4-g]benzothiazol-7-one}, protects neurons by inhibiting cyclin-dependent kinase

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Abstract

Activation of the RNA-dependent protein kinase (PKR) has been implicated in the pathogenesis of several neurodegenerative diseases. We find that a compound widely used as a pharmacological inhibitor of this enzyme, referred to as PKR inhibitor (PKRi), {8-(imidazol-4-ylmethylene)-6H-azolidino[5,4-g]benzothiazol-7-one}, protects against the death of cultured cerebellar granule and cortical neurons. PKRi also prevents striatal neurodegeneration and improves behavioral outcomes in a chemically-induced mouse model of Huntington's disease. Surprisingly, PKRi fails to block the phosphorylation of eIF2 α , a downstream target of PKR, and does not reduce the autophosphorylation of PKR enzyme immunoprecipitated from neurons. Furthermore, neurons lacking PKR are fully protected from apoptosis by PKRi demonstrating that neuroprotection by this compound is not mediated by PKR inhibition. Using *in vitro* kinase assays we investigated whether PKRi affects any other protein kinase. These analyses demonstrated that PKRi has no major inhibitory effect on pro-apoptotic kinases such as the c-Jun N-terminal kinases (JNKs), the p38 MAP kinases and the death-associated protein kinases (DAPKs), or on other kinases including c-Raf, MEK1, MKK6 and MKK7. PKRi does, however, inhibit the activity of certain cyclin-dependent kinases (CDKs) including CDK2 and CDK5 both *in vitro* and in LK-treated neurons. Consistent with its inhibitory action on mitotic CDKs, the treatment of HT-22 and HEK293T cell lines with PKRi sharply reduces the rate of cell cycle progression. Taken together with the established role of CDK activation in the promotion of neurodegeneration, our results suggest that PKRi exerts its neuroprotective action by inhibiting cyclin-dependent kinases.

Keywords

PKR; eIF2 α ; Apoptosis; Cerebellar granule neuron; Neuroprotection

Introduction

Neurodegenerative diseases place a tremendous burden on patients and health care resources. A characteristic feature of a variety of different neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and prion disease, is protein misfolding and aggregation (reviewed in Kopito, 2000; Hol *et al.*, 2006; Lansbury & Lashuel, 2006). It has been hypothesized that a contributing factor in the formation of the abnormal aggregates in neurodegenerative disorders is sustained endoplasmic reticulum (ER) stress and ER

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dysfunction (reviewed in Forman *et al.*, 2003; Shastri, 2003; Lindholm *et al.*, 2006; Zhang & Kaufman, 2006). Among the molecules activated by ER stress is the double-stranded RNA-dependent protein kinase (PKR), a kinase that was initially discovered as a key mediator in the cellular response to viral infection. Binding of double-stranded viral RNA to PKR causes its autophosphorylation, an essential step in its activation. A target of PKR is eukaryotic initiation factor 2 (eIF2 α), and the phosphorylation of eIF2 α leads to the inhibition of protein synthesis. In addition to its anti-viral action, however, PKR is known to regulate other cellular processes such as differentiation, growth, and apoptosis (Chong *et al.*, 1992; Meurs *et al.*, 1993; Lee & Esteban, 1994; Salzberg *et al.*, 2000; Kuyama *et al.*, 2003; Morimoto *et al.*, 2005; Garcia *et al.*, 2007).

A number of recent studies have implicated PKR in the pathogenesis of neurodegenerative diseases. For example, PKR activity is elevated in the brains of patients with AD, PD, HD and ALS (Peel *et al.*, 2001; Chang *et al.*, 2002; Hu *et al.*, 2003; Onuki *et al.*, 2004; Bando *et al.*, 2005). Other laboratories have reported that primary cortical neurons from PKR knockout mice are less susceptible to β -amyloid (A β) toxicity, whereas neurons and neuroblastoma cell lines overexpressing a dominant-negative form of PKR display reduced cell death in response to A β treatment (Chang *et al.*, 2002).

Recently a small-molecule chemical inhibitor of PKR has become commercially available. *In vitro* experiments conducted by Jammi *et al.* (2003) revealed that this compound, {8-(imidazol-4-ylmethylene)-6H-azolidino [5, 4,-g] benzothiazol-7-one}, designated as PKR inhibitor (PKRi), acts in an ATP-competitive manner to block autophosphorylation of PKR and PKR-dependent translation in rabbit reticulocyte lysates. We reported that PKRi prevented the death of cultured cerebellar granule neurons induced by potassium deprivation (Chen *et al.*, 2008). Similar protection was observed by Shimazawa and Hara (2006) in the neuroblastoma SH-SY5Y cell-line following treatment with tunicamycin, an agent that induces ER stress, and more recently by Wang *et al.* (2007) in cerebellar granule neurons induced to die by treatment with amprolium, a compound that causes thiamine deficiency. These results raise the exciting possibilities that chemical inhibitors of PKR, such as PKRi, may have therapeutic value in the treatment of neurodegenerative diseases.

In this report we analyzed the neuroprotective effects of PKRi in more detail. We find that besides protecting cultured cortical and cerebellar granule neurons from apoptosis, administration of PKRi prevents neurodegeneration and improves behavioral outcome in a chemically-induced mouse model of HD. Surprisingly, neuroprotection by PKRi is not mediated by the inhibition of PKR. Instead, PKRi is a potent inhibitor of cyclin-dependent kinases (CDKs) and glycogen synthase kinase (GSK) *in vitro*, two kinases that promote neuronal death in a variety of *in vitro* and *in vivo* paradigms of neurodegeneration (reviewed in D'Mello & Chin, 2005). Our results indicate that PKRi protects neurons by suppressing the activity of specific cyclin-dependent kinases.

MATERIALS AND METHODS

Materials

All cell culture media and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Unless indicated otherwise, all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). PKRi was purchased from Calbiochem (La Jolla, CA, USA). Antibodies used in this paper were as followed: anti-Phospho-eIF2 α (9721S) and anti-active caspase 3 (9661S) were from Cell Signaling Technology (Beverly, MA, USA); anti-PKR(B-10, sc-6282), anti-ATF-3(C-19, sc-188), anti-cyclin A(J-3, sc-6247), anti-CDK5(C-19, sc-596) and anti-CDK2(D-12) (sc-6248) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Tubulin (T5326) and anti-BrdU (B8434) were from Sigma-

Aldrich (St. Louis, MO, USA); Ki67 (RM-9106) was from Lab Vision Corporation (Fremont, CA, USA). Fluorescence conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA). Radioactive materials were from MP Biomedicals (Solon, OH, USA) including [γ - ^{32}P] ATP and [^{32}P] orthophosphate.

Cell culture

Animals used in this paper were treated in accordance with the Guidelines of NIH. Cerebellar granule neurons were cultured from 7-day-old Wistar rats which were treated in accordance to the Guidelines of NIH, as described by D'Mello *et al* (1993) in Basal Minimal Eagle (BME) medium containing 10% FBS, 25mM KCl, 2 μM glutamine and 0.2% gentamycin and plated on poly-L-lysine coated dishes (1 X 10⁶ cells/well in 24-well dish and 12 X 10⁶ cells/dish in 60mm dishes). 18–22 hours after plating, arabinofuranosylcytosine (AraC) (10 μM) was added to the culture medium to prevent proliferation of non-neuronal cells. Cortical neurons were cultured from neocortex of embryonic day 17 (E17) Wistar rat embryos (Murphy *et al.*, 1990). Dissociated neurons were cultured in Minimum Essential Medium (MEM) containing GLUTAMAX and supplemented 10% FBS at densities of 8 X 10⁵ cells/well in 24-well dish and 4X 10⁶ cells/dish in 35mm dishes on poly-L-lysine coated dishes. The HT-22 and 293T cell lines were purchased from ATCC (Manassas, VA, USA). HT-22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (without sodium pyruvate) supplemented with 10% FBS, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. 293T cells were cultured with complete DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Treatment of neuronal cultures

The treatment of CGNs was performed 6–7 days after plating. Apoptosis was induced by switching the cultures to LK medium (BME containing glutamine and gentamycin with 5mM KCl). PKRi was added at the same time the neuronal medium was switched. Control CGNs were treated in HK medium (BME containing glutamine and gentamycin with 25mM KCl). Viability was quantified 24 hours after treatments as described previously. Apoptosis of cortical neurons was induced one day after plating using 1mM HCA (pH7.5) or 2–3 days after plating using 5 μM camptothecin (CPT). PKRi was also added at the same time with either HCA or CPT treatment. Viability was quantified 24 hours after HCA treatment or 6 hours after CPT treatment.

Evaluation of cell viability

Viability of neurons was quantified by diamidino-2-phenylindole hydrochloride (DAPI) staining, which permits the visualization of condensed or fragmented nuclei as previously described (D'Mello *et al.*, 1993). Briefly, cultured neurons were fixed using 4% paraformaldehyde for 15 minutes on ice. Neurons were washed once with phosphate buffered saline (PBS, pH 7.4) after fixation and stained with DAPI (0.1 $\mu\text{g}/\text{ml}$ in PBS) for 10 minutes. Neurons with nuclei condensation or fragmentation were scored as apoptotic cells.

The viability of HT-22 cells was evaluated by propidium iodide (PI) staining, the Terminal Uridine deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay and active caspase-3 immunocytochemistry. PI and TUNEL staining were performed 15 hours after PKRi treatment. 1 $\mu\text{g}/\text{ml}$ PI was added to culture 30 minutes before fixation of the cells with 4% paraformaldehyde. TUNEL assay was performed using DeadEndTM Fluorometric TUNEL System from Promega (Madison, WI, USA) according to the manufacturer's instructions. Active caspase 3 immunocytochemistry (1: 200 dilution) was performed 6 hours after PKRi treatment. Cells were fixed and treated with 0.2% Triton for 5 minutes and then blocked with 5% BSA and 5% goat serum in PBS for 30 minutes. The primary

antibodies were incubated with the cells overnight at 4°C. After three washes with phosphate-buffered saline (PBS), the cells were incubated with secondary antibodies for 45 minutes at 25°C after which the cells were washed with PBS. To visualize nuclei, cells were stained with DAPI for 15 minutes at 25°C. Cell number of HT22 cells was also quantified using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Briefly, HT-22 cells were plated into 24-well tissue culture dishes at 3×10^4 cells per well one day before PKRi treatment. After PKRi treatment, cell number was determined 24 hours later by the MTT assay as previously described (Koulich *et al.*, 2001).

Genotyping of PKR^{-/-} mice and culturing of neurons

PKR^{-/-} mice were provided by Dr. John C. Bell from the University of Ottawa, Ottawa, Canada. As previously described (Abraham *et al.*, 1999), the mice are fertile and display no obvious phenotype. Mutant mice were bred and litters genotyped. Cerebellar granule neurons were cultured from the mutant mice as described above for rat cultures.

3-nitropropionic acid (3-NP) injection and behavior test

3-NP administration and analysis of brain sections were performed as previously described (Chin *et al.*, 2004). Briefly, 3-NP was injected to mice intraperitoneally at 50mg/kg twice a day and 0.27 mg/kg PKRi once a day for 4 days. After finishing the injection, we performed the behavioral test by using Tru-Scan[®] activity monitoring system (Coulbourn Instruments, PA, USA). The following behavioral parameters were selected: (i) Total movements time (ii) Total movement distance (iii) Average movement per distance and (iv) Mean velocity. Next, the mice were deeply anesthetized. Then, the brains were removed, washed in PBS and fixed in 4% paraformaldehyde overnight. Next day, they were transferred to 20% sucrose over night and rapidly frozen in Cryo-Stat embedding medium (StatLab Medical Products, Lewisville, TX, USA). 40 micron coronal sections were cut on a cryostat and stained with cresyl violet to visualize the brain architecture (Chin *et al.*, 2004).

Protein preparation and Western blot analysis

Cells were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and proteins were collected by centrifugation at 14,000 rpm for 10 minutes. After the concentration of protein was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), protein from each sample was normalized and boiled with 6X SDS loading buffer [375 mM Tris-HCl (pH 6.8 at 25°C), 12% SDS, 60% glycerol, 300 mM dithiothreitol (DTT), 0.012% bromophenol blue] at 95°C for 5 minutes. Next, the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electronically transferred to (polyvinylidene difluoride) PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After Ponceau S staining to confirm equal protein transfer, the membrane was blocked with blocking buffer [5% milk and 0.1% Tween in Tris-Buffered Saline (TBS)]. The PVDF membrane was then probed with primary antibody diluted in either blocking buffer or bovine serum albumin (BSA) buffer (1% BSA and 0.1% Tween in TBS) at 4°C overnight. The antibody dilutions were 1:1,000, except tubulin which was used at 1:10,000. After 3 washes with TBS-T (0.05% Tween in TBS), the PVDF membrane was probed with horseradish peroxidase-conjugated secondary antibodies with dilution 1:10,000 at 25°C for 1 hour. Western Blot detection was enhanced *via* chemiluminescence (ECL) kit from GE Health Care Life Science (Piscataway, NJ, USA).

³²P-metabolic labeling on endogenous PKR

60mm dishes of 7-day-old neurons were washed twice with warm, phosphate-free BME and incubated in phosphate-free BME containing 25 mM KCl for 4 hours. Next, the cultures were then incubated for 3 hours in HK, LK or LK plus PKRi media containing 250μCi/ml

[^{32}P] orthophosphate. After being lysed in ice-cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4 , 50 mM NaF, 30 mM β -glycerophosphate, 1 mM EDTA, protease inhibitors mixture), the lysates were subjected to immunoprecipitation by using PKR antibody (5 μl) and the products of immunoprecipitation were resolved by SDS–PAGE and transferred electrophoretically to PVDF membrane. After the transfer, labeled proteins were visualized by autoradiography using a Storm860 scanner (Amersham Biosciences, Piscataway, NJ, USA). Data were quantified using ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA) (Liu & D’Mello, 2006).

Kinase profiling

Kinase profiling was performed using the KinaseProfiler Service from Millipore (Billerica, MA, USA) on a fee for service basis. In short, 5–10mU of purified kinase was used along with an appropriate quantity of synthetic substrate in buffer containing optimal amount of [γ - ^{32}P] ATP for each kinase with or without 100 nM PKRi. Next the reaction mix was incubated at room temperature for 40 minutes. Then, it was stopped using a 3% phosphoric solution, spotted, washed and dried for scintillation counting.

Immunoprecipitation and CDK kinase assay

Whole cell lysates from HT-22 cells or neurons were incubated with 5 μl of primary CDK2 or CDK5 antibody and 20 μl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. Immunoprecipitates were collected by centrifugation at 6000 rpm for 30 seconds and washed twice with cell lysis buffer and twice with kinase buffer (40 mM Tris-HCl pH 7.5, 8 mM MgCl_2 , 50 mM β -glycerol phosphate, 1 mM DTT). Histone H1 (Millipore, Billerica, MA, USA) was added as a substrate in kinase buffer supplemented with 50 μM ATP and [γ - ^{32}P] ATP for 30 minutes at 30°C. The kinase reactions were stopped by addition of 6 $\times$ SDS sample buffer and boiled at 95°C for 5 minutes. Proteins were resolved by SDS–PAGE and transferred electrophoretically to PVDF membrane. Later, PVDF membrane was subjected to autoradiography. Histone H1 was detected by using Ponceau S staining to confirm equal amount of substrate loaded.

Evaluation of cell proliferation by BrdU and Ki67 staining

3×10^4 HT-22 cells and 5×10^4 293T cells per well were plated one day before PKRi treatment. For BrdU assay, 20 μM BrdU was added 24 hours after PKRi treatment. After BrdU incorporation with cells for 2 hours, HT-22 cells were fixed with 4% paraformaldehyde in PBS and washed 3 times using 0.1M PBS containing 0.5% Triton. Then, the cells were incubated with 1N HCl for 10 minutes on ice, 2N HCl for 30 minutes at 37°C, 0.1 M borate buffer (pH 8.5) for 12 minutes at room temperature, and blocked with 0.1M PBS containing 1% BSA and 5% goat serum for 15 minutes. Immunostaining was performed by incubation with a monoclonal BrdU antibody (1:200 dilution) overnight at 4°C. The secondary antibody was incubated for 45 minutes at 25°C. Cell nuclei were stained with DAPI for 15 minutes at 25°C. For Ki67 staining, immunocytochemistry was performed as described above for active caspase 3 staining. The Ki67 antibody was used at 1:200 dilution.

RESULTS

PKRi is neuroprotective in primary cultured neurons

Primary cultures of cerebellar granule neurons undergo apoptosis when switched from medium containing an elevated level of potassium (HK) to medium containing non-depolarizing levels of potassium (LK) (D’Mello *et al.*, 1993). Treatment with PKRi

completely prevents neuronal apoptosis in this paradigm (Chen *et al.*, 2008; also Fig. 1). To examine if this neuroprotection is restricted to cerebellar granule neurons and to LK-induced apoptosis, we evaluated the efficacy of this compound in primary cortical cultures induced to die by treatment with homocysteic acid (HCA), a stimulus that causes oxidative stress (Murphy *et al.*, 1990; Ratan *et al.*, 1994a; 1994b). As shown in Fig. 2A & 2C, substantial neuroprotection was also observed in this paradigm. A similar protection against HCA-induced cell death was observed in the hippocampally derived HT-22 cell line (Supplemental Fig. 1). Camptothecin (CPT) is a DNA damaging agent which induces apoptosis in cultured cortical neurons (Morris & Geller, 1996). As shown in Fig. 2B & 2C, PKRi is protective in this paradigm as well. Thus, PKRi is an effective and versatile neuroprotective agent in tissue culture paradigms.

PKRi protects against neurodegeneration and improves behavioral outcome in a mouse model of Huntington's disease

Administration of mice with 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase, results in a Huntington's disease-like pathology which includes selective striatal neurodegeneration and movement deficits (Beal, 1993; Brouillet *et al.*, 1999). We examined whether PKRi was effective in this *in vivo* disease model. As shown in Fig. 3A, administration of 3-NP causes a substantial amount of cell loss in the striatum as evidenced by the lack of cresyl violet staining. Co-administration of PKRi at 0.27 mg/kg of body weight protected against 3-NP-induced striatal degeneration. Moreover, mice that were co-administered with 3-NP and PKRi also displayed significantly better locomotor performance as compared with mice receiving 3-NP alone (Fig. 3B).

PKRi does not inhibit PKR in neuronal cultures

A downstream substrate of PKR is eukaryotic initiation factor eIF2 α (Clemens & Elia, 1997). As shown in Fig. 4A, treatment of cerebellar granule neurons with LK resulted in a small increase in eIF2 α phosphorylation compared to HK-treated cultures. Unexpectedly, the addition of PKRi to LK-treated cultures does not reduce the increase in eIF2 α phosphorylation (Fig. 4A). Given that three other kinases PERK, HRI, and GCN2, can also phosphorylate eIF2 α , it is possible the increased phosphorylation in LK is not mediated by PKR, but by one of the other kinases. Arguing against such a possibility, however, is our observation that 2-aminopurine (2-AP), another selective inhibitor of PKR, has been used in several *in vitro* and *in vivo* studies (Hu & Conway, 1993; Cheshire *et al.*, 1999; Ruvolo *et al.*, 2001; Ben-Asouli *et al.*, 2002), does inhibit the LK-mediated increase in eIF2 α phosphorylation (Fig. 4A).

We extended our studies to cortical neurons treated with either homocysteic acid or camptothecin. Although potent inducers of apoptosis, neither homocysteic acid nor camptothecin treatment increases the extent of eIF2 α phosphorylation (Fig. 4B). Co-treatment with PKRi actually increases the extent of eIF2 α phosphorylation (Fig. 4B), despite its reported inhibitory effect on purified PKR enzyme *in vitro* (Jammi *et al.*, 2003). The lack of an inhibitory effect on eIF2 α phosphorylation in both cerebellar granule neurons and cortical neurons suggests that PKRi is incapable of inhibiting PKR in cultured cells (Fig. 4B).

Because eIF2 α phosphorylation is only an indirect indicator of PKR activation, we performed a more direct assay. Activation of PKR requires its autophosphorylation and this phosphorylation of PKR has been used to evaluate its activation (Lasky *et al.*, 1982; Galabru & Hovanessian, 1987). Using ³²P-metabolic labeling and immunoprecipitation, we find that PKR autophosphorylation increases in LK-treated cultures (Fig. 4C) consistent with the

pattern of eIF2 α phosphorylation. Again, PKRi fails to inhibit the increase in PKR autophosphorylation.

Neuroprotection by PKRi does not involve PKR

Biochemical analysis failed to detect an inhibitory effect of PKRi on the activity of PKR, but the possibility our compound protects neurons by blocking some aspect of PKR function could not be completely excluded. To investigate this issue we cultured neurons from mice lacking PKR. Genotyping and expression analysis confirmed mice from which the neuronal cultures were generated lacked a functional PKR gene (Fig. 5A & 5B). As shown in Fig. 5C, LK-treatment of neurons from PKR^{-/-} mice induces apoptosis demonstrating that PKR is not necessary for LK-induced neuronal death. More importantly, treatment with PKRi completely protects PKR^{-/-} neurons from LK-induced death. This finding demonstrates PKRi acts through a PKR-independent mechanism and points to a molecule other than PKR as the target of its neuroprotective action.

Kinase profile of PKRi

PKRi is a 3' substituted indolone. Crystallographic studies have demonstrated that 3' substituted indolones are ATP-competitive inhibitors of protein kinases with the selectivity towards a specific kinase depending on the substituents on the indolone core, especially at the C-3 position (Mohammadi *et al.*, 1997; Sun *et al.*, 1998; Sun *et al.*, 2000; Davis *et al.*, 2001). Since PKRi does not activate or act through PKR, its neuroprotective effect likely involves the inhibition of another kinase. It is noteworthy that although Jammi *et al.* (2003) demonstrated PKRi could inhibit PKR activity *in vitro*, its selectivity against other kinases was not tested in that study or in any other study since. To investigate this possibility we evaluated the effect of PKRi on different pro-apoptotic kinases including the c-Jun N-terminal kinases (JNKs), stress-activated protein kinases (SAPKs or p38 MAP kinases), GSK3 kinase, and death-associated protein kinases (DAPKs) was evaluated. The effect of PKRi on select CDKs was also examined because inappropriate activation of cyclin-dependent protein kinases (CDKs) has also been shown to induce apoptosis in a variety of neuronal systems (reviewed in Copani *et al.*, 2007; Greene *et al.*, 2007). As shown in Fig. 6, PKRi highly inhibits CDK1, CDK2 and CDK5. Significant inhibition of GSK3 α and GSK3 β was also observed. In contrast, the drug inhibited JNK3, CDK6 and MKK6 less effectively and failed to inhibit other pro-apoptotic kinases included in our analysis. Likewise, PKRi had no inhibitory effect on a number of other kinases including c-Raf, MEK1 and MKK7 in these *in vitro* assays. These results suggest that the neuroprotective action of PKRi could be mediated by inhibition of either GSK3 or cyclin-dependent kinases. Consistent with this notion are reports from a number of different laboratories demonstrating that pharmacological inhibition of either of these kinases is protective against neurodegeneration both in cell culture paradigms as well as *in vivo* models of neurodegeneration (reviewed in Bhat *et al.*, 2004; D'Mello & Chin, 2005; Copani *et al.*, 2007; Greene *et al.*, 2007).

PKRi is a CDK inhibitor

We and other laboratories have shown that neuronal apoptosis is accompanied by an activation of GSK3 α and GSK3 β (Hetman *et al.*, 2000; Chen *et al.*, 2004; Chin *et al.*, 2005; Enguita *et al.*, 2005). The activation of GSK3 β requires dephosphorylation of its serine residues 9 and 21 (and in the case of GSK3 α at homologous residues). As observed previously (Chin *et al.*, 2005; Johnson *et al.*, 2005; Shimazawa *et al.*, 2007; Wang *et al.*, 2007), treatment with LK leads to a reduction in GSK3 α/β phosphorylation reflective of the increase in its kinase activity. Not only does PKRi not inhibit the LK-induced reduction of GSK3 α/β phosphorylation, but the drug reduces the extent of GSK3 α/β phosphorylation even more than seen with LK alone (Fig. 7). The inability of PKRi to inhibit GSK3 activity

in neurons indicates that GSK3 α/β inhibition is not the mechanism by which PKRi protects neurons.

We next focused on CDK2 because it was the most potently inhibited of the mitotic CDKs. The ability of PKRi to inhibit the activity of the CDK2 complex immunoprecipitated from cerebellar granule neurons was tested in an *in vitro* kinase assay. As shown in Fig. 8A, the addition of PKRi to immunoprecipitated neuronal CDK2 inhibits its activity. To examine whether PKRi could inhibit CDK2 activity in intact neurons, we added PKRi to neuronal cultures and evaluated the effect on kinase activity after immunoprecipitation of the enzyme from the cultures. As shown in Fig. 8B, CDK2 activity is slightly elevated in LK-treated neuronal cultures as compared with cultures treated with HK. When added to LK medium, PKRi inhibits CDK2 activity to a level that is even lower than that observed in HK.

To confirm these results in an independent model system, we used HT-22 cells, which as we have described above (Supplemental Figure 1), are protected by PKRi against HCA-toxicity. As shown in Fig. 9A, treatment of this neuroblastoma cell line with PKRi reduces the activity of CDK2. The reduced activity is not because of a decrease in the expression of CDK2 or of associated cyclin A (Fig. 9B & 9C).

Our results demonstrate that PKRi inhibits CDK2 activity but do not establish that this action effectively inhibits cell cycle progression. To investigate this issue, we examined the effect of PKRi on the proliferation of HT-22 cells. Treatment of HT-22 cultures with PKRi results in a decrease in total cell number when examined 24 hours after addition of the drug (Fig. 10A). To rule out the possibility that the reduced cell number is due to toxicity we performed propidium-iodide staining. As shown in Fig. 10B (left panel), no increase in propidium iodide staining was observed in the presence of PKRi. We used TUNEL-staining (Fig. 10B, middle panel) and active-caspase-3 immunocytochemistry to detect cell death (Fig. 10B, right panel). No increase in the extent of cell death was observed in the presence of PKRi. The results of the three cell death assays eliminate toxicity as being responsible for the reduced cell numbers.

To more directly examine the effects of PKRi on cell proliferation we measured BrdU-incorporation, a commonly-used assay of cell proliferation. As shown in Fig. 11A & 11B, the rate of cell cycle progression was substantially reduced in HT-22 cells treated with PKRi. Similar results were obtained with roscovitine, a well-characterized CDK inhibitor (Meijer, 1996; Rudolph *et al.*, 1996; De Azevedo *et al.*, 1997; Meijer *et al.*, 1997). The inhibition of cell cycle progression by PKRi was confirmed by the almost complete absence of Ki-67 immunoreactivity (Fig. 11B). Ki-67 is a protein that is expressed during all active phases of the cell cycle and is a widely used proliferation marker (Scholzen & Gerdes, 2000). Similar results were obtained using HEK293T cells (Fig. 11B) and in the striatal N1E115 cell-line (data not shown).

DISCUSSION

Our laboratory has previously reported that a number of different chemical compounds containing a 3' substituted indolone core can protect neurons from apoptosis (Johnson *et al.*, 2005). Among the 3' indolones we tested more recently was PKRi, a compound sold as an inhibitor of PKR and used by many as such (Shimazawa & Hara, 2006; Eley *et al.*, 2007; Eley & Tisdale, 2007; Ito *et al.*, 2007; Russell *et al.*, 2007; Wang *et al.*, 2007). PKRi is impressive in its ability to prevent LK-induced apoptosis of cerebellar granule neurons (Chen *et al.*, 2008). In contrast to many other 3' substituted indolones which display toxicity at doses that are 5 – 10 times higher than their neuroprotective concentrations, PKRi is not toxic even when used at very high doses (Chen *et al.*, 2008). We report that this compound

can also prevent the death of primary cortical neurons caused by homocysteic acid, a compound that produces oxidative stress. Additionally, PKRi inhibits apoptosis caused by the DNA damaging agent, camptothecin, in cortical neuron cultures. Most importantly, PKRi is beneficial in a chemically-induced mouse model of Huntington's disease. Our previous (Chen *et al.*, 2008) and current findings along with the findings from other groups published recently (Shimazawa & Hara, 2006; Shimazawa *et al.*, 2007; Wang *et al.*, 2007) demonstrate that PKRi is an effective and versatile neuroprotective agent that could have value in the treatment of neurodegenerative diseases. It may be noted however, that although capable of reducing 3-NP induced striatal degeneration rather substantially, PKRi administration only partially improved locomotor activity in 3-NP treated mice. The reason for this is not clear but likely due to non-selective effects of PKRi on cells and organ systems outside the nervous system. Lethal actions of this compound when administered at high doses to rats has been reported recently (Ingrand *et al.*, 2007). We have also noticed that when administered to adult mice at doses of 0.7 mg/kg body weight or higher, PKRi is generally lethal. Given these serious side effects, structural modifications of PKR are necessary before it can be considered for pre-clinical testing.

PKRi was identified by Jammi *et al* (2003) in a screen of a small library of 26 different ATP-binding site directed chemical inhibitors. The screen was performed *in vitro* using a kinase domain-containing fragment of human PKR expressed and purified from bacteria as a GST-fusion protein. Since its discovery, PKRi has been used in a number of studies to inhibit PKR (Shimazawa & Hara, 2006; Eley *et al.*, 2007; Eley & Tisdale, 2007; Ito *et al.*, 2007; Russell *et al.*, 2007; Wang *et al.*, 2007). A surprising finding of our study is that in cultured cerebellar and cortical neurons, PKRi does not inhibit PKR discernibly as judged by its inability to reduce eIF2 α phosphorylation and PKR autophosphorylation. On the contrary, in cortical neurons PKRi treatment stimulates eIF2 α phosphorylation. A similar stimulatory effect of PKRi on eIF2 α phosphorylation was observed in cerebellar granule neurons and Hela cells treated with tunicamycin, an agent that induces endoplasmic reticulum stress (data not shown). To conclusively rule out the possibility that PKRi acted by inhibiting PKR, we utilized neuronal cultures generated from mice lacking PKR. We discovered that PKRi was completely effective in protecting these cultures from cell death indicating that a molecule other than PKR was the target of PKRi at least in the context of neuroprotection.

The selectivity of PKRi against kinases other than PKR has not been reported previously. Because it did not inhibit PKR in cultured neurons and because PKR was not necessary for the neuroprotective effect of PKRi, we tested whether this compound inhibited other pro-apoptotic kinases. We discovered that PKRi inhibits CDK1, CDK2, and CDK5 potently when tested against purified enzyme. We confirmed that PKR inhibits CDK2 in intact neurons and when added to CDK2 complex immunoprecipitated from neuronal cultures. Furthermore, the treatment of different cell lines with PKRi reduces their ability to proliferate. Increased expression of cyclins and activation of CDKs has been reported in a number of different paradigms *in vivo* and *in vitro* of neurodegeneration (Herrup & Busser, 1995; Yang *et al.*, 2001). Neuroprotection through the inhibition of specific CDKs using molecular approaches as well as pharmacological agents has been amply demonstrated (D'Mello & Chin, 2005; Copani *et al.*, 2007; Greene *et al.*, 2007). It is therefore likely that PKRi exerts its neuroprotective action through its anti-CDK activity. Although the efficacy of PKRi can be explained entirely on the basis of its anti-proliferative action, it is possible that its inhibitory effect on other kinases also contribute to neuroprotection. For example, PKRi inhibits the non-mitotic CDK5 kinase strongly when tested *in vitro*. We have confirmed that the inhibition of CDK5 by PKRi also occurs in intact cerebellar granule neurons (Supplemental figure 2). Activation of CDK5 has previously been implicated in promoting neuronal death in several experimental paradigms, including cerebellar granule

neurons (Jorda *et al.*, 2005; Verdaguer *et al.*, 2005a; Verdaguer *et al.*, 2005b). Further studies are necessary to examine whether inhibition of CDK5 contributes to PKRi-mediated neuroprotection, and if so, to what extent.

It is well established that the activation of c-Jun is a central event in the promotion of neuronal death (Estus *et al.*, 1994; Ham *et al.*, 1995; Herdegen *et al.*, 1998; Watson *et al.*, 1998; Crocker *et al.*, 2001). More recently we and others have reported that ATF-3 induction is also a common feature of neuronal death both *in vivo* and *in vitro* (Tsujino *et al.*, 2000; Averill *et al.*, 2004; Ohba *et al.*, 2004; Holtz *et al.*, 2006; Huang *et al.*, 2006; Chen *et al.*, 2008). In cerebellar granule neurons, siRNA-mediated suppression of ATF-3 has a protective effect (Tsujino *et al.*, 2000; Averill *et al.*, 2004; Ohba *et al.*, 2004; Holtz *et al.*, 2006; Huang *et al.*, 2006; Chen *et al.*, 2008). We find that PKRi completely inhibits the apoptosis-regulated increase of ATF-3 expression in cerebellar granule neurons as well as cortical neurons. A similar inhibition of c-Jun activation by this compound was previously reported (Chen *et al.*, 2008). Our results suggest that the induction of c-jun and ATF-3 expression in neuronal apoptosis is connected to the activation of cell cycle components and attempted reentry into the cell cycle. Although not much is known about the relationship between ATF-3 and the cell cycle, studies using proliferating cell types have shown that c-Jun stimulates cell cycle progression by inducing expression of cyclin D, E2F, and cyclin A (Vanharä *et al.*, 2007; Shen *et al.*, 2008). Inhibition of the CDK inhibitor p21^{WAF1/CIP1} by c-Jun has also been recently reported (Kolomeichuk *et al.*, 2008; Stepniak *et al.*, 2008). These findings place c-Jun activation upstream of CDK activation. Our study indicates that PKRi inhibits CDK2 directly. At least in neurons, therefore, inhibition of CDK2 activity leads to the suppression of apoptosis-associated c-Jun activation. The molecular mechanism by which CDK inhibition leads to reduced c-Jun or ATF-3 expression is unclear. It is also conceivable, however, that the inhibition of c-Jun and ATF-3 by PKRi is a distinct effect that is unconnected to CDK2 inhibition.

In summary, our study provides three important conclusions - (1) PKRi is an effective and versatile neuroprotective agent, a modified form of which (given its toxicity in mice at high doses) may have value in the treatment of human neurodegenerative disorders; (2) The neuroprotective action of this compound is not mediated by the inhibition of PKR, and (3) PKRi inhibits CDKs 1, 2, and 5 and blocks proliferation of cells in culture. In view of the established efficacy of a number of other structurally diverse CDK inhibitors to prevent neuronal death, the neuroprotective effect of PKRi is likely to be due to its action against CDKs. Our findings suggest that caution must be exercised when using PKRi as an inhibitor of PKR in cultured cells and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3-NP	3-nitropropionic acid
BrdU	Bromodeoxyuridine

CPT	Camptothecin
CDK	Cyclin-dependent kinase
DAPI	Diamidino-2-phenylindole hydrochloride
ER	Endoplasmic reticulum
eIF2α	Eukaryotic initiation factor 2
GSK	Glycogen synthase kinase
HCA	Homocysteic acid
HK	High potassium
LK	Low potassium
PKR	Double-stranded RNA-dependent protein kinase
PKRi	PKR inhibitor
PI	Propidium iodide
TUNEL	Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

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Figure 1. PKRi is neuroprotective in cerebellar granule neurons

6 - 7 days after plating, cerebellar granule neurons were switched to HK (control), LK or LK medium containing 10 μM PKRi. Viability was quantified by DAPI staining after 24 hours treatments. Percentage of survival was normalized to the control.

(A) Data shown above represent the mean values of viability with their corresponding standard deviations from three independent experiments. * indicates statistical significance between LK and LK+PKRi values (p-value < 0.05). Statistical analysis was performed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test.

(B) *Top row*: the cell morphology of cerebellar granule neurons was observed by phase contrast microscope 24 hours after the treatments of HK, LK or LK medium containing 10 μM PKRi. *Bottom row*: DAPI stained nuclei were observed under fluorescence microscope. Neurons displaying nuclear condensation or fragmentation were quantified as apoptotic.

Figure 2. PKRi is neuroprotective in cortical neurons

(A) Cultured cortical neurons were untreated (Un, control), treated with 1mM HCA or 1mM HCA containing 10μM PKRi. Viability was quantified by DAPI staining after 24 hours treatment. Percentage of survival was normalized to the untreated control. **Data shown above represent the mean values of viability with their corresponding standard deviations from three independent experiments. * indicates statistical significance between HCA and HCA+PKRi values (p-value < 0.05). Statistical analysis was performed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test.**

(B) Cultured cortical neurons were untreated (Un, control), treated with 5μM CPT or 5μM CPT containing 10μM PKRi. Viability was quantified by DAPI staining 6 hours after treatment. Percentage of survival rate was normalized to the control. **Data shown above represent the mean values of viability with their corresponding standard deviations from three independent experiments. * indicates statistical significance between CPT and CPT+PKRi values (p-value < 0.05). Statistical analysis was performed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test.**

(C) The morphology of nuclei in cortical neuron cultures was examined 24 hours after HCA treatments or 6 hours after CPT treatments. The proportion of cells with condensed or fragmented nuclei was quantified as apoptosis.

Figure 3. PKRi prevents striatal lesion and locomotion dysfunction mediated by 3-NP

(A) Mice were injected with 3-NP, 3-NP + PKRi or saline (Control) twice a day for 4 days. After the last day of injection, whole brains removed and 40 μ m coronal sections were stained with cresyl violet. 3-NP injected animals display selective striatal degeneration as evidenced by reduced cresyl violet staining. The extent of 3-NP-induced degeneration is reduced with PKRi.

(B) 3-NP treated mice present significant locomotion dysfunction, including total movement time, total movement distance, average distance per movement and mean velocity. PKRi injection improves these locomotor activities of mice comparing with 3-NP injection alone. **Data shown above represent the mean values of movements with their corresponding standard deviations from seven animals. * indicates statistical significance between 3-NP and 3-NP+PKRi values (p-value < 0.05). Statistical analysis was performed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test.**

Figure 4. PKRi does not inhibit PKR efficiently in neurons

(A) Cerebellar granule neurons were treated with HK, LK, LK medium containing 10mM 2-AP or 10 μ M PKRi for 3 hours. Lysates from these cultures were subjected to Western blot using a phospho-eIF2 α antibody. The same membrane was re-probed with tubulin to ensure equal loading.

(B) Cortical neurons were treated with 1mM HCA or 1mM HCA plus 10 μ M PKRi for 6 hours and 5 μ M CPT or, 5 μ M CPT plus 10 μ M PKRi for 4 hours. Untreated (Un) and treated lysates were made and subjected to Western blot. eIF2 α phosphorylation and ATF-3 expression were determined using phospho-eIF2 α and ATF-3 antibodies. The same membrane was re-probed with tubulin to show equal loading.

(C) Cerebellar granule neuron cultures metabolically labeled with [32 P] orthophosphate in HK, LK or LK medium containing 10 μ M PKRi. Lysates were made and PKR was immunoprecipitated. The autophosphorylation of PKR was determined by autoradiography after SDS-PAGE and membrane transferring. The same membrane was also probed with PKR antibody to show equal amount of PKR pull down. Kinase activity was quantified by densitometric analysis of the bands and normalized to HK. The histograms represent the mean of kinase activity with **standard deviations from 3 independent experiments.**

Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 5. PKRi protects neurons lacking PKR from LK-induced apoptosis

(A) Genotyping of PKR^{-/-} and wild-type PKR^{+/+} mice. Prior to culturing of neurons, the genotypes of the mice were confirmed by PCR.

(B) Western blot analysis of cerebellar granule neurons cultured from 7-day old PKR^{-/-} and wild-type (PKR^{+/+}) mice confirm absence of PKR in the cultures from knockout mice. The same membrane was re-probed with tubulin to show equal loading.

(C) Complete protection by PKRi in PKR^{-/-} neuronal cultures. 7-day old cerebellar granule neurons cultured from mice were treated with HK (Control), LK, or LK medium containing 10 μ M PKRi. Cell viability was quantified 24 hours later. **Data shown above represent the mean values of viability with their corresponding standard deviations from three independent experiments. * indicates statistical significance between LK and LK + PKRi values (p-value < 0.05). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison Test.** *Upper panel:* Phase contrast micrograph and fluorescence micrograph of neurons treated with HK, LK, or LK + PKRi (10 μ M). *Lower panel:* Quantification of neuronal viability by DAPI-staining. The data represents the mean values of viability with standard deviation.

Figure 6. Kinase profile of PKRi

The kinase activity of each kinase was performed *in vitro* with 100nM PKRi. The optimal concentration of ATP was used for each kinase. The data shows the percentage of relative kinase activities compared with control (same kinase but without PKRi treatment). The histogram represents the mean of relative kinase activity with standard deviation. * indicates the p-value < 0.05 and ** indicates **p-value < 0.01** using Student's t-test comparing with untreated kinase reactions.

Figure 7. PKRi treatment activates GSK3 in neurons

Cerebellar granule neurons were treated with HK, LK or LK medium containing 10 μ M PKRi for 3 hours and lysates from these cultures subjected to Western blotting analysis using a phospho-GSK3 α/β antibody. The same membrane was re-probed with tubulin to ensure equal loading.

Figure 8. PKRi inhibits CDK2 in neurons

CDK2 was immunoprecipitated from cerebellar granule neurons that were treated as described below. Kinase activity was assayed in an *in vitro* reaction containing [γ - 32 P] ATP and using Histone H1 as substrate. Relative activity was visualized by autoradiography. Ponceau S staining of Histone H1 shows that comparable amounts of substrate were used in each reaction. An aliquot of the lysates prior to immunoprecipitation was also analyzed by Western blotting using tubulin antibody to demonstrate that similar quantities of lysate was used for pull-down.

(A) Cerebellar granule neurons were treated with LK for 9 hours. CDK2 was immunoprecipitated and an *in vitro* kinase assay performed in the presence (+) or absence (-) of 1 μ M PKRi.

(B) Cerebellar granule neurons were treated with HK, LK or LK containing 10 μ M PKRi for 9 hours. Lysates were made and CDK2 was immunoprecipitated and used in an *in vitro* kinase assay.

Figure 9. PKRi inhibit CDK2 kinase activity in HT-22 cells

(A) HT-22 cells were untreated (Un) or treated with 10 μ M PKRi for 3 hours. Lysates were made and CDK2 kinase assay was performed with the Histone H1 as the substrate. The kinase activity was determined by autoradiography. Histone H1 from Ponceau S staining to demonstrate that comparable amounts of substrate were used in each reaction. An aliquot of the lysates prior to immunoprecipitation was also analyzed by Western blotting using tubulin antibody to demonstrate that similar quantities of the lysates were used for pull-down.

(B) 3h untreated (Un) or treated 10 μ M PKRi whole cell lysates were harvested from HT-22 cells. Western blot analysis was performed using cyclin A antibody. The same membrane was re-probed with CDK2. Expression of CDK2 was not changed by PKRi. Western blot using tubulin antibody shows equal amount of protein loading.

(C) HT-22 cells were untreated (Un) or treated with 10 μ PKRi for 3 hours. Cyclin A was co-immunoprecipitated from these lysates using CDK2 antibody. IgG was used as control (Ctrl). Western blot was performed using Cyclin A antibody to show the amounts of cyclin A pulled down. The same membrane was re-probed with CDK2 to show equal amounts of CDK2 were immunoprecipitated. Western blot using tubulin antibody show equal amount of input protein was administrated before immunoprecipitation.

Figure 10. Reduced HT-22 cell proliferation by PKRi is not associated with drug toxicity
 (A) HT-22 cells were treated with PKRi at the indicated doses and the MTT assay utilized to evaluate the number of cells in the dish. Data was normalized to untreated cells (Control). **Data shown above represent the mean values of proliferation rates with their corresponding standard deviations from three independent experiments. * indicates statistical significance between control and PKRi treatment values (p-value < 0.05). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test.**

(B) HT-22 cells were untreated (Un), treated with 10μM PKRi or 1mM HCA. HCA treatment was used as a positive control for cell death. *Left:* PI staining was performed to detect cell death 15 hours after treatments. PI positive cells show red fluorescence. DAPI staining allows for determination of apoptotic condensed and/or fragmented nuclei. *Middle:* TUNEL assay was performed 15 hours after treatments. DAPI staining provides the morphology of nuclei. *Right:* After 6 hours treatments, active caspase 3 immunoreactivity is shown in red. DAPI staining provides the morphology of nuclei.

Figure 11. PKRi inhibits HT-22 cell proliferation and DNA synthesis

(A) HT-22 cells were treated with increasing concentrations of PKRi as indicated. BrdU incorporation assay was performed 24 hours after PKRi treatment to evaluate the rate of cell cycle progression. **Data shown above represent the mean values and standard deviations of BrdU positive rates with standard deviations from three independent experiments. * indicates statistical significance between control and PKRi treatment values (p-value < 0.05). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test.**

(B) HT-22 and 293T cells were untreated (Un), treated with 10μM PKRi or with 50μM Roscovitine (Ros) for 24 hours. The BrdU or Ki67 positive cells were visualized by performing immunocytochemistry with antibodies against BrdU or Ki67. Texas Red-conjugated secondary antibody was used to detect the immunoreactivity. DAPI staining was performed to visualize the morphology of cell nuclei.