Double-Stranded RNA-Dependent Protein Kinase-Dependent Apoptosis Induction by a Novel Small Compound

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ABSTRACT

The interferon-induced, double-stranded RNA-dependent protein kinase (PKR) can play critical roles in inhibiting virus replication and inducing apoptosis. To develop new agents that may inhibit viral replication or induce apoptosis in cancer cells via the PKR signaling pathway, we screened a chemical library for compounds that have differential cytotoxic effects on wild-type [mouse embryonic fibroblast (MEF)/PKR(+/+) and PKR-knockout [MEF/PKR(−/−)] mouse embryonic fibroblast cells. We identified a synthetic compound, BEPP [1H-benzimidazole-1-ethanol,2,3-dihydro-2-imino-a-(phenoxymethyl)-3-[(4-chlorophenyl)methyl]-monohydrochloride; DMSO, dimethyl sulfoxide; JNK, c-Jun NH2-terminal kinase; SRB, sulforhodamine B; PBS, phosphate-buffered saline; TCID50, median tissue culture infective dose; MOI, multiplicity of infection; Akt, protein kinase B; PACT, a protein activator of double-stranded RNA-dependent protein kinase.]

which leads to dimerization and autophosphorylation of PKR (Williams, 2001). Activated PKR phosphorylates the α subunit of protein synthesis initiation factor eIF2α, leading to inhibition of protein synthesis and eliciting antivirus and antitumor activities. Moreover, PKR has been reported to induce apoptosis by modulating activities of eIF2α (Der et al., 1997), nuclear factor κB (Gil et al., 1999), activating transcription factor-3 (Hai and Hartman, 2001), and p53 (Garcia et al., 2006). Because of this, activation of PKR in tumor cells has been proposed as a modality for cancer treatment. We and others (Pataer et al., 2002; Emdad et al., 2007) have found previously that adenoviral-mediated overexpression of the melanoma differentiation-associated gene 7 induced apoptosis in cancer cells by activation of PKR. Blocking PKR activation inhibited adenoviral-mediated overexpression of the melanoma differentiation-associated gene 7-induced apoptosis, suggesting a critical role of PKR activation in such apoptosis. Likewise, PKR plays an essential role in...
apoptosis induced by tumor necrosis factor (Yeung et al., 1996) and oncolytic viruses (Gaddy and Lyles, 2007). Furthermore, tumor-specific activation of PKR by dsRNA molecules induced apoptosis of glioblastoma cells in vitro and suppressed glioblastoma in vivo (Shir and Levitzki, 2002). It is interesting that expression and autophosphorylation of PKR were increased in several types of cancer, including melanoma, colon cancer, and breast cancer (Kim et al., 2000, 2002).

The fact that PKR plays an important role in apoptosis induction and that its expression is increased in several types of cancers led us to hypothesize that a balance between increased PKR expression and increased antiapoptosis molecules may be established in cancer cells, preventing them from undergoing apoptosis, and that small molecules that can break this balance might be useful for selective induction of apoptosis in cancer cells that overexpress PKR. To search for such small compounds, we used PKR wild-type [PKR(+/+)] and PKR-knockout [PKR(−/−)] mouse embryonic fibroblasts (MEFs) to screen a chemical library from ChemBridge Research Laboratories, Inc. (San Diego, CA) for chemicals that can effectively kill PKR(+/+) MEFs but not PKR(−/−) MEFs. We found that the synthetic compound BEPP can induce cytotoxic effects more effectively in MEF/PKR(+/+) cells than in MEF/PKR(−/−) cells. The apoptosis induction by BEPP, including caspase activation and BAX overexpression, was PKR dependent and was abrogated when PKR activation was blocked with the dominant-negative PKR. Furthermore, we found that BEPP can dramatically suppress Vaccinia virus replication. Thus, BEPP could be a useful PKR inducer and antivirus agent.

Materials and Methods

Cell and Culture Conditions. The PKR wild-type MEFs [MEF/PKR(+/+)] and PKR-knockout MEFs [MEF/PKR(−/−)], provided by Dr. Glen Barber (University of Miami, Miami, FL), have been described previously (Yang et al., 1995). The human cervical cancer cell line HeLa and the human lung cancer cell lines H1299, H460, H226B, and A549 were maintained in our laboratory. The human bronchial epithelial (HBE) cell line was purchased from Clonetics (Walkersville, MD). All cells except for HBE cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and maintained in the presence of 5% CO2 at 37°C. HBE cells were cultured in serum-free keratinocyte medium (Invitrogen, Carlsbad, CA).

Chemicals and Antibodies. A chemical library with 10,000 compounds, including BEPP and its analog BECC, was obtained from ChemBridge Research Laboratories, Inc. The chemicals in the library were provided at a concentration of 5 mg/ml in dimethyl sulfoxide (DMSO). The chemical structure of BEPP is shown in Fig. 1. The compound was dissolved in DMSO to a concentration of 10 mM and stored at 4°C as a master stock solution. Antibodies to the following proteins were used for Western blot analysis: BAX, Bel-2, PKR, cyclin D1, and caspase-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phosphorylated-PKR (Millipore, Billerica, MA); eIF2α, phosphorylated eIF2α, phosphorylated JNK, phosphorylated p38, AKT, and phosphorylated AKT (Cell Signaling Technology Inc., Danvers, MA); and β-actin (Sigma-Aldrich, St. Louis, MO).

Cytotoxicity Studies. The viability of the cell lines was determined using the sulforhodamine B (SRB) assay, as described previously (Pauwels et al., 2003). Cells (2–8 × 104 cells in 100 μl culture medium/well) were seeded in 96-well flat-bottomed plates and treated the next day with the agents at the indicated concentrations.

After the indicated times, cells were fixed with trichloroacetic acid. The protein was stained with sulforhodamine B, and the optical density at 570 nm was determined. Relative cell viability was determined by setting the viability of the control cells (exposed only to DMSO) at 100% and comparing the viability of the treated cells with that of the controls. The experiments were performed at least three times for each cell line.

Flow Cytometric Analysis. For analysis of the intracellular DNA content, floating and attached cells treated with BEPP were harvested, washed twice in PBS, and fixed in 70% ethanol at 4°C overnight. For detection of apoptosis, fixed cells were suspended in PBS containing 10 μg/ml propidium iodide (Roche Diagnostics, Indianapolis, IN) and 10 μg/ml RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell cycle analysis was performed using an Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA) with MultiCycle software (Phoenix Flow Systems, San Diego, CA). Accumulation of sub-G1 cells, a known indicator of DNA fragmentation and apoptosis, was used to quantify apoptosis. All experiments were repeated at least thrice.

Western Blot Analysis. For preparation of whole-cell extracts, cells were washed twice in cold PBS, collected, and lysed in lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) containing 1× proteinase-inhibitor cocktail (Roche Diagnostics). The lysates were spun at 14,000g in a microcentrifuge at 4°C for 10 min, and the resulting supernatants were used as whole-cell extracts. Protein concentrations were determined by using the BCA protein assay kit (Pierce Chemical, Rockford, IL). Equal amounts (30–50 μg) of proteins were used for immunoblotting as described previously (Terniushi et al., 2003).

Viruses and Titer Analysis. The oncolytic Vaccinia virus also has been described previously (Guo et al., 2005). VSP is a modified Vaccinia virus that has deletions in two antiapoptosis serpin genes, SPI-1 and SPI-2 (Guo et al., 2005). Adenoviruses expressing the dominant-negative dsRNA-dependent protein kinase gene (Ad/PKRΔ6) were provided by Dr. Abujiang Pataer (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The expansion, purification, titration, and quality analysis of both vectors were performed at the Vector Core Facility at M. D. Anderson Cancer Center as described previously (Fang et al., 1998). The titer used for Vaccinia virus in this study was the infectious units determined by the 50% tissue culture infectious dose (TCID50) assay (Fang et al., 1998).

Statistical Analysis. Differences among the treatment groups were assessed by analysis of variance using StatSoft statistical software (StatSoft, Tulsa, OK); p < 0.05 was regarded as significant.

Results

Library Screening for Compounds with Differential Cytotoxic Effects for MEF/PKR(+/+) and MEF/PKR(−/−) Cells. We used the MEF/PKR(+/+) and MEF/PKR(−/−) cell lines to screen for compounds that have differential effects on cell growth because of cellular PKR status. Cells seeded in 96-well plates in parallel were treated with each compound at a final concentration of approximately 5 μg/ml. Cells treated with DMSO (final concentration 1% of DMSO) at 100% and comparing the viability of the treated cells with that of the controls. The experiments were performed at least three times for each cell line.

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Results

Library Screening for Compounds with Differential Cytotoxic Effects for MEF/PKR(+/+) and MEF/PKR(−/−) Cells. We used the MEF/PKR(+/+) and MEF/PKR(−/−) cell lines to screen for compounds that have differential effects on cell growth because of cellular PKR status. Cells seeded in 96-well plates in parallel were treated with each compound at a final concentration of approximately 5 μg/ml. Cells treated with DMSO (final concentration 1%) were used as controls. A pilot experiment showed that 1% of DMSO did not have obvious impact on viability of the MEF cells. Changes in cell morphology were then observed under a microscope, and cell viability was determined by the SRB assay 2 to 4 days after treatment. The compounds that were initially observed to have differential effects on MEF/PKR(+/+) and MEF/PKR(−/−) cells underwent two additional screenings to confirm the observation. From 3000 compounds screened, we identified a compound (BEPP) that suppressed the growth of MEF/PKR(+/+) cells more effectively than that of MEF/PKR(−/−) cells (Fig. 1A). BEPP killed MEF/PKR(+/+)
cells with an IC₅₀ value of 1.4 M, whereas its IC₅₀ value on MEF/PKR(+/−) cells was approximately 17.4 M (Fig. 1, B and C), approximately 10-fold higher than the value for MEF/PKR(+/+).

**Induction of Apoptosis in MEF/PKR(+/−) by BEPP.** To investigate whether the BEPP-mediated difference in cell viability between MEF/PKR(+/+) and MEF/PKR(+/−) cells was caused by suppression of cell growth or by induction of apoptosis, we examined the caspase activation in MEF/PKR(+/+) and MEF/PKR(+/−) cells treated with BEPP. Cells were treated with various concentrations of BEPP for 72 h, and cell lysates were subjected to 8 to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with caspase-3-specific antibodies. Cleavage of caspase-3 was easily detected at concentrations of 2.5 and 10 M in MEF/PKR(+/+) cells but not in MEF/PKR(+/−) cells, indicating that BEPP can induce apoptosis in MEF/PKR(+/+) but not MEF/PKR(+/−) cells at these concentrations. Western blot analysis of BAX and Bcl-2 showed that, at a high concentration (10 M), BAX expression was increased, whereas Bcl-2 expression decreased in MEF/PKR(+/+) cells after treatment with BEPP. The significance of those changes in BAX and Bcl-1 levels was not yet clear. However, treatment with BEPP had no detectable effect on Bax or Bcl-2 expression in MEF/PKR(+/−) cells (Fig. 2A).

The apoptosis induction by BEPP in MEF/PKR(+/+) was further confirmed by fluorescence-activated cell sorting analysis. We determined the percentage of apoptosis after MEF/PKR(+/+), and MEF/PKR(−−) cells were treated with different doses of BEPP for 72 h or after the cells were treated with 10 M BEPP for different times. Cells were then harvested for quantification of apoptotic subdiploid cells by flow cytometry (Fig. 2, B and C). At 24 and 72 h after treatment with 10 M BEPP, 32.3 and 84.6% of MEF/PKR(+/+) cells, respectively, were in sub-G₁ phase. Likewise, dramatic increases of the sub-G₁ portion in MEF/PKR(+/+) cells were observed at doses of 5 and 10 M. In contrast, only background levels (less than 5%) of sub-G₁ cells were seen in MEF/PKR(−−) cells under the same treatment conditions (Fig. 2, B and C). These results demonstrated that BEPP can induce apoptosis in MEF/PKR(+/+) in a dose- and time-dependent manner.

**PKR Activation Is Required for BEPP-Induced Apoptosis.** The induction of apoptosis observed in MEF/PKR(+/+) but not MEF/PKR(−−) cells suggested a role for PKR pathways in BEPP-induced apoptosis. Therefore, we investigated the changes in PKR, eIF2α, and cyclin D1 after BEPP treatment. Cells were treated with various concentrations of BEPP for 72 h, and then lysates were analyzed by Western blotting. Control cells were treated with DMSO. The
results showed that treatment of MEF/PKR(+/-) cells with BEPP increased the amounts of phosphorylated PKR and eIF2α, which were easily detectable at the concentration of 2.5 μM (Fig. 3). Levels of phosphorylated PKR after treatment of BEPP at the doses of 2.5 and 10 μM were increased approximately 2- and 3.5-fold, respectively, when determined by a densitometric analysis and normalized with that of β-actin (Supplemental Fig. 1). In contrast, treatment with BEPP did not result in detectable changes of phosphorylated eIF2α in MEF/PKR(-/-) cells. Cyclin D1 was decreased after BEPP treatment in MEF/PKR(+/-) cells, which was consistent with eIF2α phosphorylation because phosphorylation of eIF2α results in the inhibition of cyclin D1 translation (Brewer and Diehl, 2000). We also examined the activation of the MAPKs JNK, p38, and AKT after BEPP treatment (Fig. 3). Treatment with BEPP led to an increase in phosphorylated AKT in MEF/PKR(+/-) cells. However, changes in phosphorylated JNK did not differ significantly between MEF/PKR(+/-) and MEF/PKR(-/-) cells. Moreover, BEPP increased the amount of phosphorylation of p38, but this increase did not differ significantly between MEF/PKR(+/-) and MEF/PKR(-/-) cells.

To further investigate the role of PKR activation in BEPP-induced apoptosis, we analyzed the effect of adenovector-mediated transfer of the dominant-negative PKR (Ad/PKRΔ6), which can block PKR activation. Cells treated with Ad/LacZ were used as a mock control. MEF/PKR(+/-) cells were pretreated with 1000 MOI Ad/PKRΔ6 for 24 h and then treated with 10 μM BEPP for 72 h. Cells were harvested for fluorescence-activated cell sorting analysis to quantify apoptotic cells or for Western blotting analysis to determine phosphorylated PKR and PKR expression. Pretreatment with Ad/PKRΔ6 but not the control vector dramatically reduced BEPP-induced apoptosis. The sub-G1 percentage decreased from 84.6 to 21.2% (p < 0.01) (Fig. 4A). Moreover, pretreatment of Ad/PKRΔ6 blocked BEPP-induced PKR activation, as evidenced by marked reductions of phosphorylated PKR compared with that of Ad/LacZ-pretreated cells (Fig. 4B). This result indicates that PKR activation is critical for BEPP-induced apoptosis.

PKR-Overexpressing Cancer Cells Are More Sensitive to BEPP. We then investigated whether levels of PKR expression may affect the susceptibility to BEPP. For this purpose, we evaluated expression of PKR in the human lung cancer cell lines H1299, H460, H226B, and A549 and in normal HBE cells by Western blot analysis. The results showed that H226B cells had higher PKR expression compared with the other four cell lines (Fig. 5A). Next, we determined cell viability of these five cell lines after treatment with BEPP at various concentrations for 72 h. The result showed that H226B cells had higher PKR expression compared with the other four cell lines (Fig. 5A). Next, we determined cell viability of these five cell lines after treatment with BEPP at various concentrations for 72 h. The result showed that BEPP inhibited growth of all five cell lines in a dose-dependent manner (Fig. 5B). The IC50 values at 72 h for H226B, H460, A549, H1299, and HBE were 4.6, 11.5, 13.8, 15.8, and 15.6 μM, respectively. H226B, which has higher PKR expression than the other four cell lines, was more sensitive to BEPP. This result indicates that BEPP or its
active analogs might be useful for treatment of cancers with overexpression of PKR.

Inhibition of Vaccinia Virus Replication by BEPP. Accumulating evidence has shown that PKR activation is associated with inhibition of virus replication, including the Vaccinia virus (Lee and Esteban, 1993). We hypothesized that a PKR-activating compound might suppress virus replication. To test our hypothesis, we evaluated the effect of BEPP on Vaccinia virus replication in human cervical cancer HeLa cells. We first tested the effect of BEPP in HeLa cells. We found that a BEPP concentration of 7.5 μM was suitable for our study because no obvious cytotoxic effect was observed in HeLa cells up to this concentration (data not shown). Next, we pretreated HeLa cells with 7.5 μM BEPP for 48 h and then infected cells with vSP at a dose of 0.2 MOI. Cell lysates were harvested at different points and titrated for vaccine virus by the TCID₅₀ method. The compound BEEC, which has a structure similar to that of BEPP but showed no differential cytotoxic effect on MEF/PKR(+/+) and MEF/PKR(−/−) cells, was used as a control compound; it also showed no cytotoxic effect on HeLa cells up to a concentration of 7.5 μM (data not shown). The virus yield in the BEPP pretreatment group was much lower than that in the DMSO group or the BEEC group (p < 0.01); at days 4 to 6, the difference was approximately 100-fold (Fig. 6A). A similar result was observed when Vaccinia virus and BEPP were added to HeLa cells at the same time (Supplemental Fig. 2). We also harvested cell lysates after infection with vSP for 4 days and tested the status of PKR and eIF2α. BEPP pretreatment resulted in a dramatic increase of phosphorylated PKR and eIF2α (Fig. 6B).

Discussion

PKR is one of the well characterized molecules induced by IFN-γ. The well known downstream targets of PKR include eIF-2α and the regulatory subunit of protein phosphatase 2A, B56α. Both eIF-2α and B56α, when phosphorylated by PKR, lead to inhibition of protein synthesis, which is critical for PKR-mediated inhibition of virus replication. Although inhibition of protein synthesis may contribute to PKR-mediated apoptosis, other mechanisms of PKR-mediated apoptosis have also been reported. In addition to inhibition of protein synthesis, PKR has been reported to be important for the expression of proapoptotic genes, including Fas and Bax (Balachandran et al., 1998). PKR has also been reported to induce apoptosis by activation of Fas-associated death domain/caspase 8 (Balachandran et al., 1998), by interaction and phosphorylation of p53 and enhancing its transcriptional activity (Cuddihy et al., 1999a,b), and by modulating nuclear factorκB activities (Kumar et al., 1994; Gil et al., 1999). Our results showed that BEPP selectively inhibited cell growth and induced apoptosis in MEF/PKR(+/+) cells compared with MEF/PKR(−/−) cells. The IC₅₀ value for MEF/PKR(+/+) was approximately one tenth of that for MEF/PKR(−/−) cells. Moreover, treatment
with BEPP in MEF/PKR(+/-) cells resulted in increased phosphorylation of PKR, and ectopic expression of a dominant-negative PKR can block BEPP-induced apoptosis, demonstrating that BEPP-induced apoptosis is PKR dependent. The fact that BEPP treatment led to an increase of phosphorylated eIF2α and increased expression of Bax was also consistent with previous reports of PKR-mediated apoptosis (Der et al., 1997; Gil et al., 2002).

Treatment with BEPP resulted in an increase of phosphorylated PKR but not basal PKR, suggesting that the major mechanism of action is activating, but not induction, of PKR. PKR is known to be activated by binding with dsRNA, a vital cellular antiviral response upon viral infection. In addition to activation by virus infection, PKR is activated through various other stimuli, including cytokine, growth factor, and stress signals. It has been reported that PACT, a cellular protein, acts as a protein activator of PKR in response to diverse stress signals such as serum starvation and peroxide or arsenite treatment (Patel and Sen, 1998; Patel et al., 2000). After exposure of cells to these stress agents, PACT is phosphorylated and associates with PKR with increased affinity. Heterodimerization of PACT with PKR leads to PKR activation in the absence of dsRNA (Li et al., 2006). Moreover, PACT-mediated activation of PKR leads to enhanced eIF2α phosphorylation followed by apoptosis (Patel et al., 2000; Li et al., 2006). Whether PACT is involved in BEPP-mediated PKR activation is not yet clear. Nevertheless, our data suggested treatment with BEPP-induced apoptosis in a PKR-dependent manner, as was evidenced by activation of caspase 3 and dramatic increase of apoptotic cells determined by flow cytometric assay. However, PKR is known to play important role in other cell death mechanisms, such as autophagy (Tallóczy et al., 2002). Whether autophagy is also involved in BEPP-induced cell death remains to be determined.

A small compound that can induce PKR-dependent apoptosis may have two applications. First, such a compound may be useful for treatment of cancers with overexpression of PKR. It has been reported that PKR expression was increased in melanoma, colon cancer, and breast cancer (Kim et al., 2000, 2002). More recently, we have found that PKR played a role in radioresistance (von Holzen et al., 2007). In this study, we tested whether levels of PKR expression in human lung cancer cell lines may affect their sensitivity to BEPP. We found that H226B cells, which have high levels of PKR compared with other cell lines, were more sensitive to BEPP than were other cell lines tested. This result demonstrated that the PKR level could be a marker of cytotoxic effects for BEPP and its active analogs. Second, because most virus infection can cause activation of PKR, induction of PKR-dependent apoptosis may lead to the death of infected cells, thereby preventing replication of the virus. We found that oncolytic Vaccinia virus replication was suppressed as much as 99% by pretreatment host cells with BEPP. However, pretreatment of the Vaccinia virus with BEPP did not have any impact on initial virus infection (data not shown), suggesting that BEPP affects Vaccinia virus replication rather than infection. The finding was consistent with previous reports that PKR activation can inhibit virus replication, such as that of Vaccinia virus, HIV, hepatitis C virus, influenza virus, varicella-zoster, and herpes simplex virus (Adelson et al., 1999; Muto et al., 1999; Bergmann et al., 2000; Pflugheber et al., 2002; Desloges et al., 2005; Chang et

![Fig. 5. Cytotoxicity of BEPP in four lung cancer cell lines and in normal bronchial epithelial cells. A, total PKR expression was determined by Western blot analysis in these cell lines. B, dose-response to BEPP. Cells were treated with various concentrations of BEPP for 72 h, and cell viability was determined by using the SRB assay. Cells treated with DMSO were used as a control, and their viability was set at 100%. Each data point represents the mean ± S.D. of three independent experiments.](#)

![Fig. 6. Effect of BEPP on vSP replication in HeLa cells. A, cells were pretreated with 7.5 μM BEPP for 48 h. DMSO pretreatment was used as a control, and BECC pretreatment was used as a compound control. Cells were then infected with vSP at a dose of 0.2 MOI. Cell lysates were harvested at the indicated time points and titrated using the TCID50 method. The virus yield in the BEPP group was much lower than in the DMSO or BECC group (p < 0.01) at days 4 to 6. B, cell lysates were harvested after infection of vSP for 4 days. The lysates were analyzed for total and active (phosphorylated) PKR and eIF2α by Western blotting.](#)
al., 2006; Smith et al., 2006; Goodman et al., 2007). Nevertheless, identification of BEPP as a PKR-dependent apoptotic inducer is only the first step in developing novel PKR-targeted therapeutics. Much work remains before BEPP or its analogs can be used as antitumor or antiviral agents.

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References


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