Reversal of Bcl-2–Mediated Resistance of the EW36 Human B-Cell Lymphoma Cell Line to Arsenite- and Pesticide-Induced Apoptosis by PK11195, a Ligand of the Mitochondrial Benzodiazepine Receptor

Donna E. Muscarella,*†† Kerry A. O’Brien,*†† Ann T. Lemley,†‡ and Stephen E. Bloom*†

*Department of Microbiology and Immunology, †Institute for Comparative and Environmental Toxicology, and ‡Department of Textiles and Apparel, Cornell University, Ithaca, New York 14853

Received December 5, 2002; accepted January 27, 2003

Opening of the permeability transition (PT) pore is a central feature of apoptosis induction by chemical stress. One component of the PT pore, the mitochondrial benzodiazepine receptor (mBzR), has recently received attention for its potential role in modulating PT pore function. Specifically, antagonistic ligands of the mBzR, such as 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide (PK11195), have been shown to sensitize Bcl-2 overexpressing cells to apoptosis induction by facilitating the opening of the PT pore and the subsequent loss of mitochondrial membrane potential (∆Ψm). We examined whether PK11195 can sensitize EW36, a human B-cell lymphoma cell line that over-expresses Bcl-2, to apoptosis induction and mitochondrial depolarization by environmental chemicals including mitochondrial toxicants. We found that, although EW36 cells are refractory to apoptosis induction by antimony A, rotenone, pyridaben, alachlor, and carbonyl cyanide m-chlorophenylhydrazone (mCCCP), they are dramatically sensitized to induction of apoptosis by low concentrations of these same agents following pretreatment with PK11195. The sensitization of EW36 cells is accompanied by a rapid and extensive loss of ∆Ψm within a few hours following chemical exposure. Furthermore, using sodium arsenite, we examined the role of the c-Jun N-terminal kinase (JNK) pathway and protein synthesis in apoptosis induction in EW36. We found that, unlike untreated cells, EW36 cells treated with PK11195 no longer show an association of JNK pathway activation with apoptosis induction. Importantly, PK11195 eliminates a requirement for protein synthesis in chemically induced apoptosis in EW36 cells. These results show significant drug-mediated alteration of cell sensitivity and JNK pathway activation to environmental chemicals and mitochondrial toxicants, following ligation of the mBzR.

Key Words: apoptosis; c-Jun N-terminal kinase; arsenite; pesticide; B-lymphocyte; PK11195.

Mitochondria are now clearly established as central to the execution of stress-induced apoptotic pathways in a variety of cell types. The permeability transition (PT) pore is a multi-protein complex that spans the inner and outer mitochondrial membranes and is vital to the maintenance of mitochondrial homeostasis. Furthermore, the PT pore is believed to be a key regulator of stress-associated signals. Exposure of cells to drugs, environmental toxicants, or radiation may result in opening of the pore, the collapse of mitochondrial membrane potential (ΔΨm), disruption of the outer mitochondrial membrane, and release of apoptogenic factors, including cytochrome c. These events subsequently lead to the cleavage of specific caspases, including procaspase-9, a proximal caspase in the apoptotic pathway, and ultimately to the cleavage of caspase-3 and its substrates, including poly (ADP ribose) polymerase (PARP). The key components of the PT pore include the adenine nucleotide transporter, the voltage-dependent ion channel, cyclophilin D, mitochondrial hexokinase, and the mitochondrial, or peripheral, benzodiazepine receptor (mBzR). In addition, members of the Bcl-2 family of proteins, including Bax, Bcl-2, and Bcl-XL expressed in lymphoid cells and other tissues, are associated with the PT pore. Moreover, evidence indicates that the antiapoptotic effect of Bcl-2 is mediated, at least in part, by modulating the opening of the PT pore (Kantrow et al., 1997; Susin et al., 1996; Zanzami et al., 1996). However, the exact mechanisms regulating PT pore opening and release of apoptogenic factors are not completely understood, and may vary as a function of cell lineage and type of stress.

One component of the PT pore, the mBzR, has recently received attention as a potential pharmacological target for the sensitization of drug-resistant tumor cells to the induction of apoptosis. Importantly, a functional relationship of the mBzR with Bcl-2 is suggested by several studies showing that treatment of cells with antagonistic ligands of the mBzR reverses the antiapoptotic effects of Bcl-2 over-expression in several cell types, including those of B- and T-lymphoid lineages (Costantini et al., 2000; Hirsch et al., 1998; Larochette et al., 1999). Among the most potent of such ligands is 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide (PK11195), which binds the mBzR with high affinity. Although PK11195 alone shows little or no toxicity in cell lines examined, it effectively synergizes with a variety of...
agents, including topoisomerase inhibitors, gamma-irradiation, arsenite, Fas ligand, and ceramide, to induce apoptosis in otherwise resistant cells that overexpress Bcl-2 (Hirsch et al., 1998; Larochette et al., 1999). Furthermore, these studies show that PK11195 directly affects mitochondria, facilitating the loss of $\Delta \psi_m$ and release of apoptogenic factors, such as cytochrome c and Smac/DIABLO. However, it is not known whether PK11195-mediated reversal of Bcl-2 protection extends to environmental chemicals. Of particular interest are chemicals that interact directly with mitochondria such as pesticides, many of which inhibit specific complexes of the respiratory chain. Cytotoxicity of such chemicals appears to be regulated by PT pore-associated proteins and is, therefore, potentially modulated by antagonistic ligands of the mBzR.

We have previously described a panel of human Burkitt's lymphoma cell lines as a model of germinal center-derived B-lymphocytes. These lines show marked differential sensitivity to apoptosis induction by a variety of chemical agents including sodium arsenite, and mitochondrial toxicants including carbonyl cyanide m-chlorophenylhydrazone (mClCCP) (Muscarella and Bloom, 2002, 2003; O’Brien et al., 2001). Our studies revealed important differences in the role of stress-kinase activation and apoptosis induction between two of these cell lines, ST486 and EW36, which express low and high levels of Bcl-2, respectively (Lee and Shacter, 1997; O’Brien et al., 2001). Specifically, we found that ST486 cells show rapid mitochondrial depolarization upon treatment with mClCCP, and undergo extensive apoptosis following exposure to low concentrations of mitochondrial toxicants as well as to arsenite, in the absence of activation of the c-Jun N-terminal stress kinase (JNK) pathway. In contrast, EW36 cells are relatively resistant to chemically induced mitochondrial depolarization and apoptosis induction. For example, EW36 cells undergo apoptosis with delayed kinetics and only after exposure to relatively high concentrations of arsenite, in a manner always associated with JNK pathway activation. Thus, our previous studies suggest a differential requirement for this stress-activated signaling pathway in chemically induced apoptosis in ST486 cells compared to Bcl-2 overexpressing EW36 cells.

The main objective of the present study was to determine the possibility and extent of reversal of Bcl-2-mediated resistance of EW36 cells to mitochondrial alteration and apoptosis induced by environmental chemicals. Specifically, we determined whether treatment of EW36 cells with PK11195 could significantly sensitize them to apoptosis induction and mitochondrial depolarization by pesticides/mitochondrial inhibitors. Furthermore, we examined the influence of mBzR ligation on the requirements for JNK pathway activation and protein synthesis for apoptosis induction by arsenite, an important environmental contaminant and model chemical used to examine the contribution of various stress kinase pathways in apoptosis induction (Chen et al., 1996; Huang et al., 1999; Liu et al., 2001; Muscarella and Bloom, 2002; 2003).

**Materials and Methods**

**Chemicals and antibodies.** The chemicals used in this study were: pyridaben and rotenone, both inhibitors of complex I of the mitochondrial respiratory chain, antimycin A, an inhibitor of complex III, and mClCCP, which uncouples oxidative phosphorylation. Pyridaben, antimycin A, and rotenone are pesticides/insecticides, and the latter two are piscicides (used to manage fish populations). In addition, alachlor, an herbicide with genotoxic activity and sodium arsenite (NaAsO$_2$), a metal that inhibits mitochondrial respiration and also reacts with sulfhydryl groups of proteins, and is also genotoxic, were studied. Sodium arsenite, cycloheximide, PK11195, mClCCP, rotenone, and antimycin A were obtained from Sigma Chemical Co. Pyridaben and alachlor were obtained from Chem-service, West Chester, PA. Rabbit polyclonal antibodies specific for the 47 kD procaspase-9 protein, and its 37 and 35 kDa cleavage products, for total and phosphorylated-JNK1/2, and for c-Jun were purchased from Cell Signaling, Inc. The antibody that recognizes the 113 kDa full length PARP and the 85 kDa cleavage product (PARP; AAP-250) was purchased from Stressgen, Inc.

**Cell lines and culture conditions.** The Burkitt’s lymphoma-cell line ST486 was obtained from ATCC, Manassas, VA. The EW36 cell line was obtained from Dr. Ian T. Magrath, National Cancer Institute, Bethesda, MD. Both of the cell lines grow optimally and similarly in the same medium formulation, have doubling times of approximately 24 h, and are Epstein Barr virus-negative. The cell lines were cultured in medium RPMI 1640, (GIBCO) supplemented with 15% fetal calf serum, penicillin-streptomycin, and L-glutamine. Cells were grown at 37°C, 5% CO$_2$, and 95% humidity.

For all experiments, cultures were set up at a density of 0.3 x 10$^6$ cells/ml and allowed to grow for 24 h. After appropriate pre-treatment (i.e. cycloheximide or PK11195), cells were plated into 6 well plates, at 3 ml / well, and chemical was added. At the designated times, cells were harvested for protein immunoblotting, determination of mitochondrial membrane depolarization, or for detection of morphological apoptosis as described below.

**Cytological detection of apoptosis and necrosis with the Hoechst 33342/propidium iodide (HPI) assay.** The induction of apoptosis was analyzed using a double-fluorescence staining technique that has been extensively validated (Muscarella and Bloom, 1997; Muscarella et al., 1998). The procedure allows simultaneous detection of plasma membrane integrity by dye exclusion and apoptotic phenotypes by observing condensed, segregated chromatin in “live” cells. Briefly, cells were stained in 20 g/ml Hoechst 33342 (emitting blue fluorescence) for 15 min, at 37°C in the dark. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin in intact but shrunken cells (fluorescing blue in early stages and red later on). The apoptotic phenotype was easy to detect and discriminate from necrotic cells, which were swollen, had irregular/damaged membranes, and were PI positive. Typically, 200 cells were scored for each sample and classified as either necrotic, apoptotic, or normal/viable.

**Detection of mitochondrial toxicant-induced membrane depolarization.** Mitochondrial toxicant-induced loss in $\Delta \psi_m$ was monitored at the single-cell level using JC-1, a membrane potential sensitive probe as previously described (O’Brien et al., 2001, Reers et al., 1991). Briefly, JC-1 is taken up selectively by energized mitochondria and forms J-aggregates that emit red fluorescence upon excitation at 490 nm. Mitochondria with low $\Delta \psi_m$ (depolarized) take up little JC-1 and emit a low-level green fluorescence, indicative of the mono-meric form of JC-1.

Cultures were treated as described above. At each of the respective time points, 5 $\mu$l of JC-1 stain (from a 400 $\mu$M stock in DMSO) was added to 200 $\mu$l of cell suspension (10 $\mu$M, final concentration) taken directly out of the culture dishes (Smiley et al., 1991). Cells were then incubated with the JC-1 dye for 20 min at 37°C in the dark. Then, 20 $\mu$l of cell suspension was pipetted onto a slide that was gently mounted with a coverslip and examined using a Leitz Aristoplan microscope equipped for epifluorescence. The red-versus-green mitochondrial-specific fluorescence from JC-1 was detected using Leitz filter cube E3. The JC-1 fluorescence status in each of 200 cells was determined for each treatment sample in the study.
Statistical analysis of the data. Two hundred cells were scored per sample and data were entered into a database for subsequent analysis. Percentages (e.g., % apoptosis) were transformed by arc sine to normalize the data. A one-way analysis of variance (ANOVA) was performed on data sets from experiments including control and treatment values. If the F-statistic was significant, post hoc comparisons were made using Fisher’s LSD test.

Protein immunoblotting. Following chemical exposure for the specific period of time indicated in each experiment, cells (1 ml of culture) were collected and lysates prepared, subjected to protein immunoblotting, and quantified by densitometry as previously described (Muscarella and Bloom, 2002). For detection of phosphorylated JNK1/2, membranes were first probed with antibodies specific for the phosphorylated forms of the kinase and subsequently reprobed using antibodies that recognize JNK1/2 independent of phosphorylation status, to insure that differences in signal were due to phosphorylation of the protein and not to differences in amounts of total protein.

RESULTS

PK11195 sensitizes EW36 cells to apoptosis induced by a variety of chemical toxicants. The ST486 cell line is highly sensitive, whereas the Bcl-2 overexpressing cell line, EW36, is relatively resistant to the induction of apoptosis by a variety of drugs (Muscarella and Bloom, 2002; O’Brien et al., 2001). The present study examined the effects of PK11195 on the sensitivity of EW36 cells towards apoptosis induction by treatment with environmental chemicals, some of which directly alter mitochondrial functions (see Materials and Methods). It should be noted that while high levels of these mitochondrial toxicants may induce nonspecific membrane damage in cells resulting in necrosis, the lower concentrations used in the present study only induced apoptosis, not necrosis, in these cell lines (O’Brien et al., 2001).

An analysis of apoptosis following exposure of cells to antimycin A (Fig. 1A), mClCCP (Fig. 1B), rotenone (Fig. 1C), or pyridaben (Fig. 2A) confirmed that ST486 cells are sensitive to apoptosis induction for each mitochondrial toxicant. In contrast, EW36 cells showed a high degree of resistance to apoptosis induction. However, pretreatment of EW36 cells with PK11195 resulted in a significant increase in sensitivity to apoptosis induction by all four agents, with numbers of apoptotic cells approaching, or exceeding, those for ST486.

Sensitization of EW36 cells by PK11195 was also found, following exposure to the herbicide alachlor (Fig. 2B). In addition, treatment with PK11195 sensitized EW36 cells to a variety of chemical toxicants with different molecular targets, including sodium arsenite (refer to Fig. 5A). Moreover, as illustrated in subsequent sections, PK11195-associated sensitization was detected not only by morphological analysis of apoptosis, but also by PARP cleavage.

PK11195 sensitizes EW36 cells to loss of $\Delta \psi_m$ following exposure to mitochondrial toxicants. Using the mitochondrial membrane-sensitive fluorochrome JC-1 to assess $\Delta \psi_m$, we previously showed that exposure of cultures of ST486 cells to mitochondrial toxicants resulted in a rapid reduction in the percent of polarized cells that preceded the induction of apoptosis. In contrast, EW36 cells were shown to be resistant to mitochondrial depolarization (O’Brien et al., 2001). However,

in the present study, PK11195-treated EW36 showed rapid and extensive loss of $\Delta \psi_m$ upon subsequent exposure to 5- and 10-$\mu$M mClCCP and rotenone (Fig. 3). In the absence of PK11195, no reduction in $\Delta \psi_m$ was detected in EW36 cells following exposure to mClCCP (Fig. 3B) or to rotenone (Fig. 3C). In addition, treatment of EW36 with PK11195 alone had no significant effect on mitochondrial polarization.

EW36 cells sensitized by PK11195 undergo chemically induced apoptosis without activation of the JNK/c-Jun pathway. Unlike the above toxicants, which alone induce little or no apoptosis in EW36 cells, arsenite is potent, with the ability to
induce apoptosis in EW36. However, there are important differences in arsenite-induced apoptosis in EW36 compared to ST486 cells. First, apoptosis is induced in ST486 cells at much lower concentration of arsenite compared to EW36, with extensive apoptosis occurring at 2 μM in ST486 cells (Muscarrella and Bloom, 2002). Second, arsenite-induced apoptosis is rapid in ST486, with levels of apoptotic cells and cleaved PARP approaching 100% by 6 h (Fig. 4, t6h) whereas apoptosis induction/PARP cleavage was detected only at later time points in EW36. Third, apoptosis induced by arsenite was not associated with activation of the JNK/c-Jun pathway, illustrated here by the absence of c-Jun protein induction in ST486 cells. In contrast, apoptosis induction, PARP cleavage, and activation of the JNK/c-Jun pathway were closely correlated in EW36 cells, with c-Jun induction always preceding PARP cleavage (Fig. 4, compare t6h and t24h).

Pretreatment of EW36 cells with PK11195 resulted in substantial differences, both quantitative and qualitative, in arsenite-induced apoptosis (Fig. 5). Pretreated EW36 cells were sensitive to apoptosis induction by much lower concentrations of arsenite, with some PARP cleavage detected at 5 μM (Fig. 5A, t24h). In addition, PARP cleavage was detected earlier in PK11195-treated EW36 cultures, with low levels of cleavage detected as early as 6 h after arsenite addition (Fig. 5A, t6h).

Importantly, the close correlation between c-Jun induction and PARP cleavage that was previously detected in EW36 cells was not found in PK11195-treated cells. Despite differences in their profiles of PARP cleavage, the profiles of c-Jun induction by arsenite appeared similar between the two treatments, with

FIG. 2. Differential induction of apoptosis by the pesticides pyridaben and alachlor in EW36 cells, with and without PK11195 pretreatment. Cultures of EW36 cells (EW, black bars) treated with solvent alone, or EW36 cells pretreated with 120 μM PK11195 for 2h (EW+PK, stippled bars) were exposed to pyridaben (A) or alachlor (B) at the indicated concentrations. After 24 h cells were scored for morphological apoptosis by the H/PI fluorescence assay. The results are presented as the mean ± SEM of three experiments. *p < 0.01, statistically different compared to 0 μM controls for each treatment group.

FIG. 3. Effect of PK11195 pretreatment on the kinetics and extent of loss of mitochondrial membrane potential following exposure of EW36 cells to mitochondrial toxicants. Cultures were pretreated with 120 μM PK11195 (+PK; dashed lines) for 2h, or untreated (-PK; solid lines), (A) without further chemical addition, or (B) exposed to mClCCP (CCP) or (C) rotenone (ROT) for the indicated times. Mitochondrial membrane potential was analyzed using the potential sensitive JC-1 fluorochrome. The graphs show the percentage of cells remaining polarized (normal membrane potential) at the indicated concentrations of the toxicants (μM) in the interval of 0.5 – 8 h. Data are means ± SE from replicate cultures and two experiments.
or without PK11195, at the early time point (Fig. 5A, t6h). However, levels of c-Jun protein declined in the PK11195-treated cultures, with little protein detected by 24 h (Fig. 5A, t24h).

The similar profiles of c-Jun induction at the early time point suggested to us that PK11195 sensitized EW36 cells to apoptosis without effecting stress-kinase signaling. This was confirmed by assessing the phosphorylation of JNK1/2 (Fig. 5B). PK11195 treatment had little effect on the profile of arsenite-induced stress kinase phosphorylation in EW36 cells, with either no, or in some experiments a slight, lowering of the threshold concentration of arsenite required to activate JNK.

Together, these data indicate that PK11195-treated EW36 cells undergo apoptosis in a manner that is similar to ST486 cells—showing accelerated kinetics of apoptosis induction at relatively low concentrations of arsenite that do not activate the JNK/c-Jun pathway.

In order to determine if PK11195-sensitized EW36 cells are generally susceptible to apoptosis induction in the absence of JNK/c-Jun activation/induction, PARP cleavage and c-Jun induction by mClCCP were examined (Fig. 6A). This mitochondrial toxicant does not induce JNK phosphorylation in ST486 or in EW36 cells (O’Brien et al., 2001). Despite the profound sensitization of PK11195-treated EW36 cells to mClCCP, no induction of c-Jun protein (Fig. 6A) or phosphorylation of JNK (Fig. 6B) was detected. Similar results were found with the other chemicals, including pyridaben, alachlor, and antimycin A (data not shown).

EW36 cells treated with PK11195 do not require protein synthesis to undergo apoptosis. The association of JNK activation/c-Jun induction with apoptosis in EW36 cells suggested the hypothesis that protein synthesis is required to induce apoptosis in this cell line and that overcoming the protective effects of Bcl-2 overexpression using PK11195 eliminated this requirement. This hypothesis was tested by determining, first, whether arsenite-induced apoptosis was blocked in EW36 cells when protein synthesis was inhibited by treatment with cycloheximide, and second, if PK11195 treatment eliminated the requirement for protein synthesis in apoptosis induction.

Blocking protein synthesis with cycloheximide drastically reduced arsenite-induced PARP cleavage in EW36 cells (Fig. 7; EW compared to EW + CHX). This effect of cycloheximide would be most likely attributed to inhibition of protein synthesis, as opposed to blocking cell cycle progression, since treatment with aphidicolin, an inhibitor of DNA synthesis, had no effect on arsenite-induced apoptosis (data not shown). Importantly, cycloheximide treatment did not reduce arsenite-induced PARP cleavage in PK11195-treated cells. In fact, while EW36 cells were completely resistant to apoptosis induction by cycloheximide alone, a low level of PARP cleavage was detected in PK11195-treated cells. In addition, we found that the data obtained for cleavage of procaspase-9, an upstream
caspase in the apoptotic pathway that is associated with mitochondria, paralleled those for PARP cleavage.

**DISCUSSION**

The mBzR is approximately an 18-kDa protein that spans the outer mitochondrial membrane and is a component of the PT pore. It has diverse cellular functions, including a role in steroid biogenesis, by regulating the transport of cholesterol into mitochondria, porphyrin transport, heme biosynthesis, and anion transport (reviewed in Verma and Snyder, 1998; Casellas et al., 2002; Cotter, 2000). It also functions as an oxygen sensor regulating mitochondrial responses to reactive oxygen species (ROS) and maintaining mitochondrial $\Delta \psi_{\text{m}}$. Moreover, it has proven to be an important pharmacological target in lymphoid cells, since mBzR ligands such as PK11195 show promise as anti-inflammatory agents (Torres et al., 2000).

Recently, the mBzR has also been identified as a potential target for pharmacological sensitization of drug-resistant tumor cells, since it is upregulated in a number of hematopoietic and solid tissue malignancies (Banker et al., 2002; Decaudin et al., 2002; Venturini et al., 1998). Moreover, in at least some cases, mBzR levels correlate with levels of Bcl-2 protein (Carayon et al., 1996). A functional association of the mBzR with Bcl-2 in regulating the PT pore is now evident and is supported by studies that show that ligands of the mBzR can overcome Bcl-2 mediated resistance by directly effecting PT pore opening (Hirsch et al., 1998). Many of the published studies on sensitization via the mBzR have focused on mitochondrial effects. However, little has been reported concerning the effects of PK11195-mediated sensitization on the requirements for stress-signaling and/or protein synthesis in the engagement of apoptotic pathways.

We found that PK11195, an antagonist of the mBzR, sensitizes the Bcl-2 overexpressing the B-lymphoma cell line, EW36, to the induction of apoptosis by a variety of chemical agents. Moreover, we have characterized several important features of the PK11195-mediated sensitization process. First, PK11195 sensitizes drug-resistant EW36 cells to the induction of apoptosis by mitochondrial toxicants that either inhibit electron transport in the respiratory chain (pyridaben, rotenone, antimycin A) or uncouple oxidative phosphorylation (mClCCP). Second, PK11195 facilitates a rapid loss of $\Delta \psi_{\text{m}}$ by mClCCP and rotenone at the same toxicant concentrations that induce apoptosis in PK11195-sensitized cells. Third, PK11195 treatment sensitizes EW36 cells to a variety of chemicals with different molecular targets, but which induce apoptosis through a mitochondrial pathway (arsenite and alachlor). Lastly, we found that PK11195-sensitized EW36 cells show fundamental differences in apoptosis-related signaling compared to untreated cells, and in several respects resemble the sensitive cell line, ST486, as demonstrated by exposure to sodium arsenite. Specifically, PK11195-sensitized EW36 cells no longer show caspase-9 cleavage in the apoptotic pathway that is associated with mitochondria, paralleled those for PARP cleavage.

**FIG. 6.** Effects of PK11195 pretreatment on mClCCP-induced PARP cleavage and JNK pathway activation in EW36 compared to ST486 cells. (A) Cultures of ST486, and EW36 were treated either with solvent alone or 120 $\mu$M PK11195 (EW+PK) for 2 h prior to addition of mClCCP at the indicated concentrations. EW36 cells treated with 80 $\mu$M arsenite alone was included as a positive control. At 6 and 24 h after chemical addition, aliquots were taken and lysates were subjected to immunoblotting for PARP and c-Jun, as indicated. The 85-kD cleavage product for PARP is indicated by the arrow, and the % of cleaved PARP, as determined by densitometry, is indicated below each lane. (B) Aliquots from above cultures were collected at 2 h following chemical addition and subjected to immunoblotting for phospho-JNK followed by total-JNK1 and JNK2 (p-JNK1 / p-JNK2 and JNK1 / JNK2).

**FIG. 7.** Effects of inhibition of protein synthesis by cycloheximide on arsenite-induced PARP and caspase-9 cleavage in EW36 cells. Cultures of EW36 cells were pre-treated with solvent alone (EW36), pre-treated with 5 $\mu$g/ml cycloheximide (EW+CHX), 120 $\mu$M PK11195 (EW+PK), or both cycloheximide plus PK11195 (EW+CHX+PK) for 2h prior to the addition of arsenite at the indicated concentrations. At 24 h after chemical addition, aliquots were taken and lysates were subjected to immunoblotting for PARP and caspase-9 (CSP9), as indicated. The 85-kD cleavage product for PARP, and the 37 and 35-kD cleavage products for caspase-9 are indicated by the arrows. The % of cleaved PARP, as determined by densitometry, is indicated below each lane.
an association of JNK pathway activation with apoptosis induction, nor do they require protein synthesis to undergo apoptosis.

Arsenite provides an important contrast to the mitochondrial toxicants in that it does induce apoptosis in EW36 cells, but only at relatively high concentrations, and with slower kinetics, compared to the ST486 cell line. Importantly, we found that PK11195 lowers the threshold concentration of arsenite required to induce apoptosis to levels in EW36 cells below the level that activates the JNK pathway, and the requirement for protein synthesis is eliminated. We previously found that JNK pathway activation and protein synthesis were consistently associated with arsenite-induced apoptosis in EW36, but not ST486 (Muscarella and Bloom, 2002). Unlike EW36 cells, ST486 cells express low levels of Bcl-2 (Lee and Shacter, 1997; O’Brien et al., 2001). While JNK pathway activation and/or protein synthesis may normally be required to overcome Bcl-2-mediated protection in EW36 cells, our data show that perturbation of the Bcl-2 function with PK11195 eliminates a requirement for new protein synthesis, thus resulting in apoptosis induction that appears to be mechanistically similar to ST486.

The use of cycloheximide does not allow us to conclude that proapoptotic proteins are specifically induced by activation of the JNK pathway, although this pathway has been shown to play a central role in stress-induced apoptosis in numerous systems (Chen et al., 1996; Huang et al., 1999; Liu et al., 2001; Muscarella and Bloom, 2002). However, our data show that cycloheximide blocks arsenic-induced caspase-9 cleavage as well as PARP cleavage in EW36 cells. These data indicate that the protein synthesis is required for apoptosis-associated mitochondrial changes, as opposed to steps in the apoptotic pathway downstream of mitochondrial events.

Studies show that sensitization of various cell types to apoptosis induction by PK11195 is attributed to its ability to bind the mBzR with high affinity. Importantly, differences in the extent of PK11195 sensitization among various cell types have been observed and can be attributed to variations in the level of expression of the mBzR (Banker et al., 2002; Decaudin et al., 2002; Venturini et al., 1998) as well as to differences in PK11195 binding affinity (Banker et al., 2002). However, alternative mechanisms for PK11195-mediated sensitization, independent of the mBzR, have also been identified. For example, in acute myeloid leukemia, PK11195 blocks drug efflux mediated by the P-glycoprotein pump (Banker et al., 2002). The mBzR has also been invoked as playing an antioxidant role in protecting hematopoietic cells against oxidative damage (Carayon et al., 1996). Studies indicate that PK11195-mediated sensitization may also involve the generation of ROS—an effect occurring at relatively higher (i.e., micromolar) concentrations of the agents compared to those required to inhibit the mBzR (i.e., nanomolar; Fennell et al., 2001).

At present, we cannot formally exclude the above possibilities in PK11195-mediated sensitization of EW36 cells. However, our data argue against these mechanisms. First, if PK11195 sensitization was achieved by blocking drug efflux, we would expect to see a quantitative (i.e., lowering of the threshold concentration of chemical required to induce apoptosis), but not a qualitative difference in the apoptotic pathway used. Our data show a mechanistic difference in apoptosis induction in PK11195-sensitized EW36 cells, characterized by the elimination of a requirement for protein synthesis and loss of association of JNK/c-Jun pathway activation. Second, ROS generation is often associated with activation of the JNK/c-Jun pathway (Allen and Tresini, 2001; Droge, 2002; Lo et al., 1996). Since PK11195-treated EW36 cells are sensitized to undergo apoptosis in the absence of JNK/c-Jun pathway activation, our data argue against a direct role for ROS. However, the potential contribution of ROS in EW36 apoptosis, as assessed by more direct measurements, is indicated as a subject for further study. Our current data most strongly support a role for JNK/c-Jun and protein synthesis in EW36 apoptosis in the absence of PK11195, whereas treatment with this agent eliminates this association.

Various studies suggest several possibilities concerning a mechanism for the JNK pathway, with or without protein synthesis, in overcoming Bcl-2-mediated resistance. Phosphorylation of various sites on Bcl-2 may modulate its antiapoptotic activity. Following treatment of cells with microtubule inhibitors, JNK itself has been shown to translocate to mitochondria and phosphorylate, and presumably inactivate, Bcl-2 (Fan et al., 2000). Conversely, ceramide, a potent activator of JNK generated during cellular stress-responses, mediates the dephosphorylation of Bcl-2 at serine 70 by inducing and/or activating a specific subunit of protein phosphatase 2A (Ruvo et al., 1999). Phosphorylation at this site in Bcl-2, which is a substrate of protein phosphatase 2A, is believed to be required for its normal, antiapoptotic function. In addition, it is possible that protein synthesis results in the induction of endogenous ligands of mBzR, such as protoporphyrin IX (PPIX) (Pastorino et al., 1994; Verma et al., 1987). PPIX is generated in vivo from the heme precursor 5-δ aminolaevulinic acid and is believed to be responsible for apoptosis induction in tumor cells subjected to photodynamic therapy. Similar to PK11195, PPIX can potentiate loss of Δψm and facilitate the induction of apoptosis. We have yet to identify the specific protein(s) induced by the JNK pathway that can reverse Bcl-2-mediated resistance in EW36. However, our data show for the first time that, by effecting PT pore function, the mBzR antagonist, PK11195, can specifically overcome the need for such factors.

ACKNOWLEDGMENTS

We thank Linda Hovanec and Daniel Lee for technical assistance and Allan Eaglesham for editorial assistance. The work was supported by grants from the National Institute of Environmental Health Science (ES010815 to D.E.M.) and the USDA/NRICGP (99–35102–8238 to A.T.L. and S.E.B.).

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