The Apoptotic Effect of HA14-1, a Bcl-2-interacting Small Molecular Compound, Requires Bax Translocation and Is Enhanced by PK11195[1]

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Abstract

HA14-1 is a small molecular compound that was identified based on the structure of Bcl-2. HA14-1 interacts with Bcl-2 and inhibits the antiapoptotic effect of Bcl-2. We investigated the mechanism of HA14-1-induced apoptosis and found that HA14-1 induces translocation of Bax from cytosols to the mitochondria. Cells deficient in Bax were much more resistant to HA14-1-induced apoptosis, suggesting that Bax is required for this process. A pan-caspase inhibitor failed to inhibit the apoptotic effect of HA14-1, indicating that this is through a caspase-independent pathway. To eliminate the effect of cytosolic Bax, we incubated cell-free mitochondria with HA14-1 to study its effect on cytochrome *c* release. HA14-1 was ineffective in causing cytochrome *c* release from the purified mitochondria. However, the combination of HA14-1 and PK11195, an antagonist of peripheral benzodiazepine receptor of the mitochondria, enhanced the cytochrome *c* release by HA14-1. The combination of PK11195 and HA14-1 could therefore serve as a potentially useful approach to enhance apoptosis in cancer.

Introduction

Regulation of apoptosis is central to development, tissue homeostasis, and tumorigenesis. The Bcl-2 family apoptotic regulators are the key players in the regulation of apoptosis (1, 2). The overexpression of the Bcl-2 family is not only a poor prognostic indicator but is also related to chemotherapy resistance (3). Targeting the Bcl-2 family is therefore a potential approach for cancer therapy. One approach currently in clinical trial uses antisense oligonucleotides against Bcl-2 (4). The more direct approach is to use small molecular compounds to block Bcl-2 function. These compounds have been identified based on the structural information of Bcl-2, and they fit into a hydrophobic pocket of Bcl-2 that binds the BH3 domain. Wang *et al.* (5) first achieved this goal by identifying HA14-1. Subsequently, two other groups also identified small molecular compounds with similar properties (6, 7). Preliminary studies demonstrated that HA14-1 strongly induced apoptosis in NIH3T3, HL60, and lung cancer H1299 cell lines with an IC_{50} in the range of 10–20 μM (5). HA14-1 is a powerful tool for inhibiting the function of Bcl-2 experimentally and a useful parental compound for the future development of clinically applicable agents.

Although the structure of Bcl-2 was established, the biochemical functions of the Bcl-2 family members remain unclear. Based on the structural information, it was predicted that Bcl-2 members might form a channel (8) and regulate the opening of the mitochondrial PTP[3] complex (9). This PTP complex includes PBR, VDAC, adenine nucleotide translocator, and cyclophilin D (9).

Among the components of the PTP complex, the role of PBR has not been resolved. It is known that PBR is related to cholesterol transport into the mitochondria in steroid hormone-producing tissues such as the ovaries and adrenal glands (10). PBR is also related to tumor oncogenesis and regulation of apoptosis. PBR expression is higher in metastatic liver cancer and highly aggressive breast cancer cells than in nonaggressive cells (11, 12). Ro5-4864, an agonist of PBR, strongly protects a human lymphoblastoid cell line against tumor necrosis factor α-induced apoptosis (13). PK11195, an antagonist of PBR, facilitates the disruption of the mitochondrial transmembrane potential ($\Delta\psi$) and apoptosis induced by glucocorticoids, etoposide, doxorubicin, and γ-irradiation, even though PK11195 has no cytotoxic effect by itself (14). It is most interesting that the effect of PK11195 in facilitating apoptosis can partially reverse Bcl-2-mediated apoptosis inhibition (14). These results suggest a potential clinical application of blocking PBR to synergize chemotherapy or radiation in cancer therapy. Blocking PBR could be a novel approach for patients who develop drug resistance to the standard chemotherapy or radiation.

We investigated the mechanism of HA14-1-induced apoptosis. We found that Bax is essential to the apoptotic effect of HA14-1. When Bcl-2 is inhibited by HA14-1, Bax translocated to the mitochondria. Bax-deficient cells were much more resistant to HA14-1 than Bax-positive cells. The combination of PK11195 and HA14-1 is more potent than HA14-1 alone in both cell-free mitochondria and the cell line

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[3] The abbreviations used are: PTP, permeability transition pore; PBR, peripheral benzodiazepine receptor; VDAC, voltage-dependent anionic channel; MEF, mouse embryonic fibroblast; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PE, phycoerythrin; 7-AAD, 7-aminoactinomycin D; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BAF, Boc-aspartyl(Ome)-Fluoromethylketone.

studies. This combination provides a novel approach to induce apoptosis in treatment of human cancers.

Materials and Methods

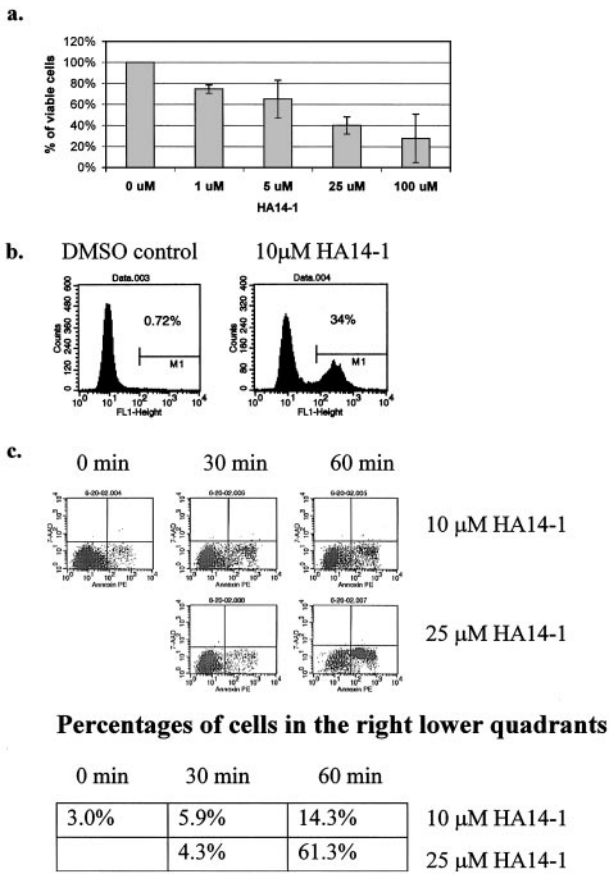
Cell Lines and Reagents. MEFs from normal and Bax−/− mice were kindly provided by Dr. Stanley Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School; Ref. 15). Human tBid plasmid was provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center). HA14-1 was purchased from Maybridge Chemical Co. (Cornwall, United Kingdom), and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) was from Sigma. Boc-D(Ome)-Fluoromethylketone was from Calbiochem (San Diego, CA). Monoclonal antibody against cytochrome *c* was purchased from BD PharMingen, and Bax antibody was from Santa Cruz Biotechnology.

Apoptosis Assays. Apoptosis assays were performed by standard TUNEL with biotin-labeled dUTP. Cells were fixed with 1% paraformaldehyde in PBS for 15 min and then fixed with 70% ethanol overnight. Cells were washed with cold PBS containing 0.125% BSA (BSA/PBS). The cell pellets that contained 2×10^6 cells were resuspended in 50 μl of enzyme solution of terminal deoxynucleotide transferase buffer, $CoCl_2$, biotin-dUTP, and terminal deoxynucleotide transferase enzyme per the manufacturer's protocol and incubated at 37°C for 30 min. Cells were washed with BSA/PBS and incubated with avidin-FITC at room temperature for 30 min in dark. Cells were again washed with BSA/PBS before fluorescence-activated cell-sorting analysis (FACS; Becton Dickinson). Alternatively, apoptotic cells were incubated with annexin V-PE along with 7-AAD (BD PharMingen) according to the manufacturer's protocol. Flow cytometry was used to quantify the percentages of annexin V-positive and 7-AAD-negative cells.

MTT Cell Viability Assays. MTT (Chemicon International Corp., Temecula, CA; Ref. 16) accumulates in viable cells and becomes blue. Absorbance reading at 570 nm has a linear correlation with the number of viable cells when the number is between 10^3 and 10^5 in each well of the 96-well plates.

Subcellular Fractionation of the Cells. HL60 cells were washed with cold PBS and treated with a buffer containing 300 mM sucrose, 10 mM Tris (pH 7.5), 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride protease inhibitor for 5 min on ice. Cells were broken by passage through 25-gauge needles for 10 strokes. The broken cells were centrifuged at 1,000 × *g*, 10,000 × *g*, and 30,000 × *g* for collection of unbroken cells/nuclei, crude mitochondria, and microsomes, respectively. The final supernatant was cytosol. Mitochondria were further purified with a sucrose gradient from 1–2 M in cell lysis buffer before *in vitro* cytochrome *c* release study.

Immunofluorescence Staining. HL60 cells were plated on slides by cytospin and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.2% Triton X-100 in PBS and blocked with 3% BSA. Immunofluorescence staining of the Bax protein was performed by standard procedure with Bax polyclonal antibody at 1:50 dilution (Santa Cruz Biotechnology). The signals of Bax were colocalized with Mito-Tracker Red (Molecular Probe).



Percentages of cells in the right lower quadrants

0 min	30 min	60 min	
3.0%	5.9%	14.3%	10 μM HA14-1
	4.3%	61.3%	25 μM HA14-1

Fig. 1. Induction of apoptosis of HL60 cells by HA14-1. *a,* dose response of HA14-1 in HL60 cells. The viable cells were determined by trypan blue exclusion. *b*, HL60 cells were treated with 10 μM HA14-1 (*right panel*) or DMSO control (*left panel*) for 4 h followed by TUNEL assays. The percentages of TUNEL-positive cells are indicated. *c,* HL60 cells were treated with 10 and 25 μM HA14-1 for 30 and 60 min and stained with annexin V-PE and 7-AAD. The percentages of apoptotic cells (annexin V-positive and 7-AAD-negative cells) are noted at the *bottom*.

Results

Induction of Apoptosis with HA14-1 in HL60 Leukemia Cells. We first performed a dose-response study of HA14-1 in HL60 leukemia cells. With a dose range from 1 to 100 μM, we observed a dose-dependent decrease in the cell numbers after 16 h of treatment with HA14-1. The IC_{50} is between 5–25 μM (Fig. 1*a*). To confirm that the decrease in cell number with HA14-1 is indeed due to apoptosis, we performed both TUNEL and annexin V-PE assays in HA14-1-treated HL60 cells to quantify the percentages of apoptosis (Fig. 1, *b* and *c*). We found that 0.72% of the DMSO-treated HL60 cells were TUNEL positive. Treatment with HA14-1 for 4 h increased the percentage of positive cells to 34%. Because TUNEL staining depends on DNA fragmentation, which is a late event of apoptosis, we used annexin V to detect the earlier apoptotic event of phosphatidylserine flipping. We found that HA14-1 induced a rapid phosphatidylserine flipping between 30 and 60 min, a time point when 7-AAD-positive cells have not appeared. By 30 min, there was no increase in annexin V-positive cells, but at 60 min after 25 μM

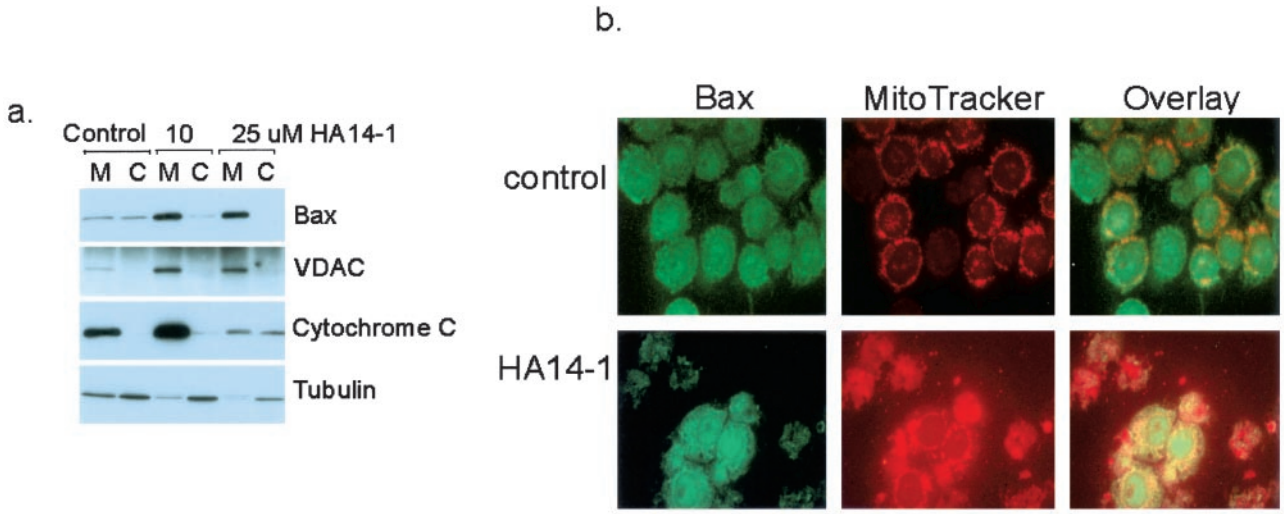


Fig. 2. Bax is translocated to mitochondria after HA14-1 treatment. *a,* HL60 cells were fractionated before and 2 h after treatment with 10 and 25 μM HA14-1. Western analysis of the mitochondria (*M*) and cytosols (*C*) was performed with Bax, VDAC, α-tubulin, and cytochrome *c* antibodies. Equal amounts of protein (20 μg by Bio-Rad protein assay) were loaded into each lane. *b,* immunofluorescence staining of Bax and colocalization with MitoTracker Red. The *top panels* are cells treated with DMSO control, and the *bottom panels* are cells treated with 25 μM HA14-1 for 2 h.

HA14-1 treatment, more than 60% of the HL60 cells became annexin V positive. This rapid time course indicated that HA14-1 induces apoptosis through a direct activation of the apoptotic pathway.

Translocation of Bax to the Mitochondria by HA14-1. We next investigated the mechanism of HA14-1-induced apoptosis. Andreeff *et al.* (17) reported that treatment with HA14-1 induces the formation of a Bax and Bcl-2 complex. This result suggests that Bax plays a role in HA14-1-induced apoptosis. We therefore investigated whether HA14-1 induces translocation of Bax from cytosols to the mitochondria. HL60 cells were subjected to subcellular fractionation, and the mitochondrial and cytosolic fractions were analyzed by Western blot with the Bax and cytochrome *c* antibodies. We found that Bax protein was present in both mitochondria and cytosols before treatment, but treatment with 10 μM HA14-1 translocated most of the cytosolic Bax to the mitochondria. Increasing the HA14-1 concentration to 25 μM moves all of the Bax protein into the mitochondria, and cytochrome *c* was also released from the mitochondria to the cytosol. Another mitochondrial protein, VDAC, was still present in the mitochondria after HA14-1 treatment (Fig. 2*a*). The Bax translocation was reconfirmed by immunofluorescence staining of the Bax protein and colocalization with a mitochondrial dye, MitoTracker Red (Fig. 2*b*). Although there was nonspecific staining in the nuclei due to cross-reaction with the Bax antibody, the Bax staining pattern had a poor overlay with MitoTracker Red dye. After 25 μM HA14-1 treatment, most of the mitochondria disintegrated due to apoptosis and thus had a diffuse staining pattern with the MitoTracker Red (Fig. 2*b*). Only a few cells still had a granular appearance by MitoTracker Red staining (Fig. 2*b*), and Bax staining had a significantly better overlay with MitoTracker Red in those cells. This is consistent with the previous findings that HA14-1 induced translocation of Bax from cytosols to the mitochondria.

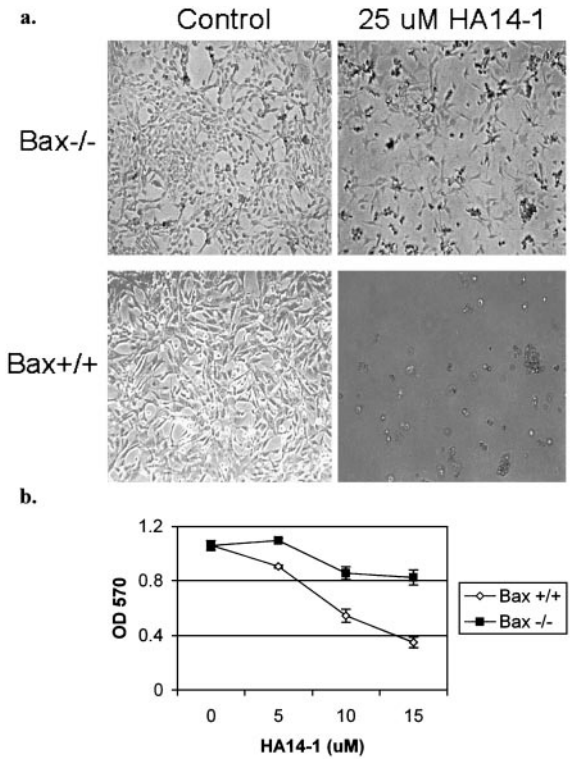


Fig. 3. Bax-deficient cells are resistant to HA14-1. *a,* phase-contrast pictures of MEFs from Bax+/+ and Bax−/− mice treated with 25 μM HA14-1 overnight. *b,* viable cells were quantified with MTT staining. Absorbance reading was performed at 570 nm.

Bax Is Required for HA14-1-induced Apoptosis. We then investigated whether the translocation of Bax is required for the apoptotic effect of HA14-1 by Bax-deficient MEFs. MEFs from wild-type mice were sensitive to 25 μM

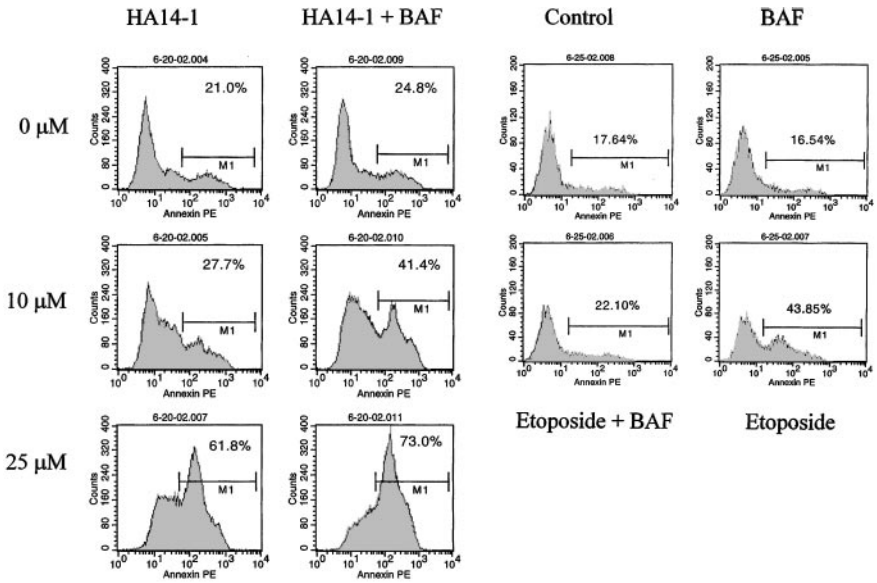


Fig. 4. HA14-1 induces apoptosis by a caspase-independent pathway. The HL60 cells were treated with 0, 10, and 25 μM HA14-1 along with the pan-caspase inhibitor BAF at 30 μM (*left panels*). A corresponding positive control was performed with 1 μM etoposide with and without same concentration of BAF (*right panels*). The percentages of apoptotic cells are quantified by annexin V-PE staining and flow cytometry.

HA14-1, similar to the HL60 cells. Treatment of Bax+/+ MEFs with same dose of HA14-1 overnight resulted in a near complete cell death by phase-contrast image. However, MEFs from Bax-deficient mice were much more resistant to HA14-1-induced apoptosis at the same dose (Fig. 3*a*). We used MTT to measure the viable cells and confirmed that Bax-deficient MEFs were more resistant to HA14-1 (Fig. 3*b*). These results indicate that Bax plays a critical role in the death effect of HA14-1.

HA14-1-induced Apoptosis Is Mediated by a Caspase-independent Pathway. To determine whether HA14-1-induced cell death is mediated through a caspase-dependent pathway, we incubated cells with BAF, a pan-caspase inhibitor, and determined whether this pan-caspase inhibitor prevents HA14-1-induced apoptosis. BAF had a weak intrinsic toxicity to HL60 cells at a concentration of 30 μM. As expected, HL60 cells were still effectively killed by 10 and 25 μM HA14-1, and 30 μM of BAF could not prevent cell death induced by HA14-1 (Fig. 4, *left panels*). A corresponding control proved that BAF at the same concentration could inhibit apoptosis induced by 1 μM of etoposide (Refs. 18 and 19; Fig. 4, *right panels*). This proves that the apoptotic effect of HA14-1 is independent of caspase activation.

HA14-1 Did Not Induce Cytochrome *c* Release from Cell-free Mitochondria in a Dose-dependent Manner. The induction of cell death is a result of dominance of the proapoptotic factor, such as Bax, over antiapoptotic factor, such as Bcl-2. In the previous results, the effect of HA14-1 was significantly weaker in Bax-deficient MEFs. This raises a very important question regarding whether neutralization of Bcl-2 alone is sufficient to trigger cell death. Because HA14-1 was identified by structural analysis of Bcl-2, it is very unlikely that HA14-1 directly interacts with Bax. Therefore, HA14-1 serves as a useful tool to examine whether neutralization of Bcl-2 is sufficient to induce apoptosis. We isolated cell-free mitochondria for incubation with HA14-1 *in vitro* to determine whether HA14-1 could induce cytochrome

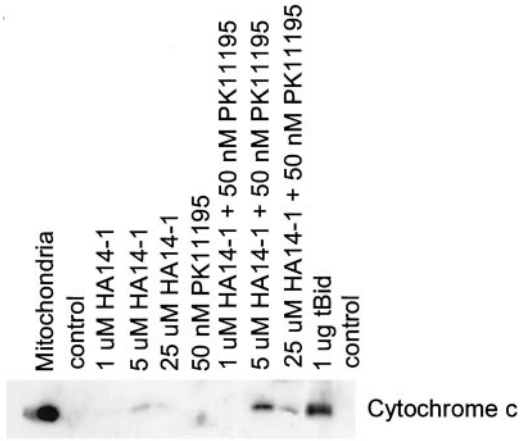


Fig. 5. HA14-1 is ineffective in inducing cytochrome *c* release from cell-free mitochondria. Purified cell-free mitochondria (100 μg) were treated with various concentrations of HA14-1 with and without 50 nM PK11195 at room temperature for 30 min. The supernatants of the incubated mitochondria were analyzed with cytochrome *c* antibody by Western blot. The positive and negative controls for cytochrome *c* release were performed using tBid recombinant protein and buffer only.

c release by itself. In this cell-free system, the contribution of Bax is eliminated because the majority of Bax is present in cytosols. The isolated mitochondria did not have any cytochrome *c* release after treatment with a detergent-free buffer that was used to dilute HA14-1. When we incubated the cell-free mitochondria with 1 or 5 μM HA14-1, we only observed a small amount of cytochrome *c* release at a dose of 5 μM. Interestingly, further increasing the dose of HA14-1 to 25 μM did not increase the amount of cytochrome *c* released as compared with treatment with 5 μM HA14-1 (Fig. 5). This was in contrast with the previous result that 25 μM HA14-1 was very effective in killing HL60 cells (Fig. 1). This result suggests that neutralization of the antiapoptotic effect of Bcl-2 alone is not sufficient to induce mitochondrial apopto-

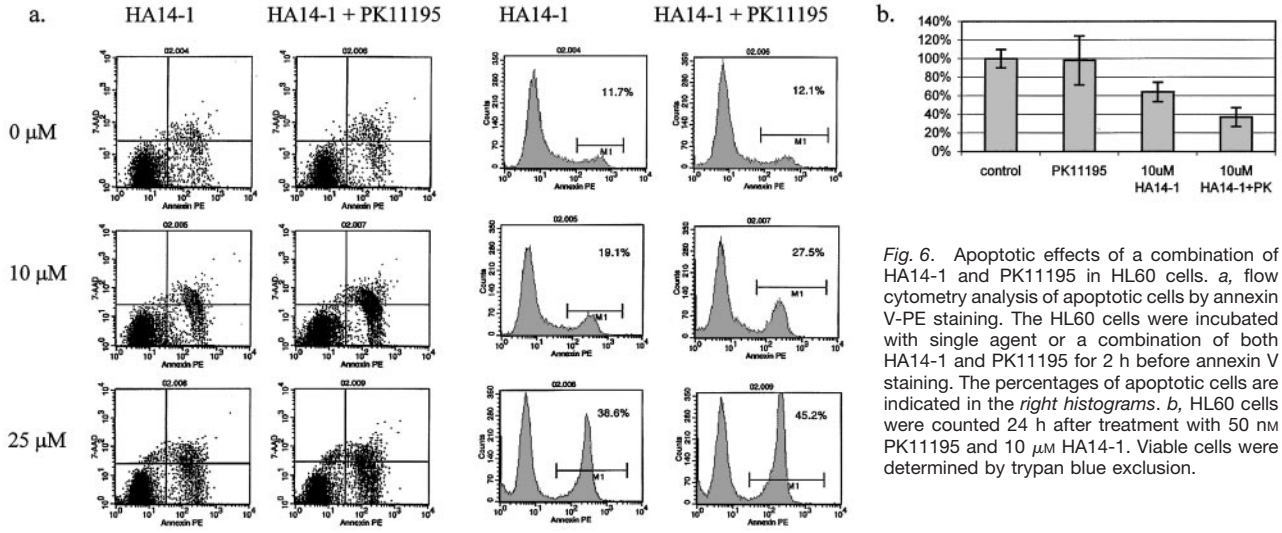


Fig. 6. Apoptotic effects of a combination of HA14-1 and PK11195 in HL60 cells. *a,* flow cytometry analysis of apoptotic cells by annexin V-PE staining. The HL60 cells were incubated with single agent or a combination of both HA14-1 and PK11195 for 2 h before annexin V staining. The percentages of apoptotic cells are indicated in the *right histograms*. *b,* HL60 cells were counted 24 h after treatment with 50 nM PK11195 and 10 μM HA14-1. Viable cells were determined by trypan blue exclusion.

sis. Other death signals, such as the translocation of Bax to the mitochondria, are essential for the triggering of cell death.

Blocking PBR Enhances Mitochondrial Cytochrome *c* Release by HA14-1. What other death signals besides Bax could induce mitochondrial cytochrome *c* release in the presence of HA14-1? We were interested in studying the effect of blocking PBR because PK11195 enhances the apoptotic effects of chemotherapeutic agents (14, 20). We tested whether the combination of HA14-1 and PK11195 induced cytochrome *c* release from the *in vitro* mitochondrial system. PK11195 by itself did not induce any cytochrome *c* release, as expected (Fig. 5, *Lane 6*), which was consistent with many other reports that PK11195 itself could not induce apoptosis (14, 21). However, the combination of PK11195 and HA14-1 enhanced the release of cytochrome *c* from the cell-free mitochondria compared with HA14-1 alone, but this release was still less than the amount of cytochrome *c* released by 1 μg of tBid (Fig. 5). This prompted us to study whether the combination of PK11195 and HA14-1 would have a synergistic effect in induction of apoptosis.

Combination of HA14-1 and PK11195 for Induction of Apoptosis. Based on the previously observed synergism between HA14-1 and PK11195 in cytochrome *c* release from cell-free mitochondria, we investigated whether the combination of HA14-1 and PK11195 is superior to HA14-1 alone for induction of apoptosis in HL60 cells. We used annexin V-PE and flow cytometry studies to quantify the percentages of apoptosis 2 h after treatment with 10 or 25 μM HA14-1 and PK11195. Based on the previous report of a high affinity between PK11195 and PBR (K_D = 10 nM), we used 50 nM PK11195. We found that 10 and 25 μM HA14-1 resulted in 19.1% and 38.6% of annexin V-PE-positive cells in HL60, respectively (Fig. 6*a*). Although PK11195 by itself did not change the percentage of cell death (11.7% *versus* 12.1%), the addition of 50 nM PK11195 to 10 and 25 μM HA14-1 increased the annexin V-PE-positive population to 27.5% and 45.2%, respectively. This enhanced effect was again confirmed by measuring the viable cells by trypan blue exclusion (Fig. 6*b*). This observation that PK11195 enhances the apoptotic effect of HA14-1 raises the possibility that the combination of PK11195 and HA14-1 is a potentially useful approach in leukemia therapy.

Discussion

In this report, we have established the mechanism of the apoptotic effect of a Bcl-2-interacting small molecule, HA14-1. HA14-1 binds Bcl-2 and neutralizes the antiapoptotic effect of Bcl-2. However, our cell-free mitochondrial study revealed that it is not enough to induce cytochrome *c* release by just blocking Bcl-2 with HA14-1. The translocation of Bax from the cytosol to the mitochondria is essential for the subsequent cytochrome *c* release. This study therefore provides an important clue regarding the primary effector in induction of cell death. Bcl-2 and Bax not only function through antagonizing each other but also have independent functions (22). Is the blocking of Bcl-2 or the activation of Bax more critical in triggering apoptosis? It has been very difficult to separate these two events due to the lack of critical tools. With the availability of HA14-1, we were able to achieve blockage of Bcl-2 in cell-free mitochondria to study the consequence of Bcl-2 blockage alone. Our result convincingly established that blocking Bcl-2 alone is not sufficient to induce cytochrome *c* release. Therefore, Bax translocation to the mitochondria is the primary effector in triggering the subsequent mitochondrial apoptotic events. A similar study by Zong *et al.* (23) also addressed this question. The authors found that BH3-only proapoptotic proteins Bim and Bad failed to induce apoptosis in the bax−/−bak−/− cells, but Bax restored the susceptibility of these bax−/−bak−/− cells to Bim- and Bad-induced apoptosis. Bax, but not Bim or Bad, was sufficient to sensitize the bax−/−bak−/− cells to a

variety of apoptotic stimuli. Our results are fully consistent with their finding that neutralization of Bcl-2 by a BH3 peptide or a small molecular compound such as HA14-1 is not sufficient to trigger mitochondrial apoptotic events. In other words, Bax is the true effector of apoptosis.

HA14-1 represents a parent compound for the future development of small molecular compounds through inhibition of Bcl-2. However, the current study raises the possibility that cancer cells lacking Bax may fail to respond to HA14-1. It is not clear whether HA14-1 could interact with and inhibit Bcl-xL. It is less likely that a universal compound could be developed to antagonize most of the Bcl-2 family cell death antagonists due to their sequence variations. With the redundancy of many cell death regulators, it remains to be seen whether the approach of blocking Bcl-2 by a compound such as HA14-1 would be useful. The genomic instability of cancer has frequently precluded the possibility of using one single target for cancer treatment. However, HA14-1 or its derivatives will at least provide a novel approach to enhance apoptosis induced by combination therapy. This is supported by the recent meeting report by Milella *et al.* (24), who demonstrated a synergism between HA14-1 and a mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitor, PD184352. It is also interesting that HA14-1 has the ability to enhance many compounds, such as PK11195 and PD184352, that are nontoxic by themselves.

What can we learn biologically from the fact that a combination of PBR inhibitor PK11195 and HA14-1 increased HA14-1-induced apoptosis, but PK11195 alone did not? It suggests that there are two checkpoints that cells use to prevent the opening of the mitochondrial PTP complex. One is the more powerful Bcl-2 family, and the other is the weaker PBR. These two checkpoints of Bcl-2 and PBR are also independent of each other but could be synergistic (25). As shown by the study of Hirsch *et al.* (14), blocking PBR can partially reverse the inhibition of apoptosis by Bcl-2. Due to the weak effect of PBR in controlling the mitochondrial PTP complex, this effect of PBR has been overlooked for a long time. We have demonstrated here that the effect of PBR could only become obvious when the antiapoptotic effect of Bcl-2 is blocked by HA14-1. The combination of HA14-1 and PK11195 was more potent than HA14-1 alone. Whether this novel approach can be translated to a clinical application as a new regimen remains to be seen.

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