Phenylarsine Oxide Induces Apoptosis in Bax- and Bak-Deficient Cells through Upregulation of Bim

Biyun Ni1,2, Qi Ma1,2, Baowei Li1,2, Lixia Zhao1,2, Yong Liu1,2, Yushan Zhu3, and Quan Chen1,3

Abstract

Purpose: Bax and Bak are regarded as key mediators for cytochrome c (Cyt c) release and apoptosis. Loss of Bax or Bak is often reported in human cancers and renders resistance of these cancerous cells to chemotherapy. Here, we investigated that phenylarsine oxide (PAO) could induce Bax/Bak-independent apoptosis.

Experimental Design: Annexin V/propidium iodide (PI) staining, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, and caspase activation assays were conducted to detect apoptosis in Bax/Bak-deficient mouse embryonic fibroblasts (MEF) and HCT116 bax−/−/c0/c0 colorectal cancer cells. Cyt c release and Bim expression were assessed by Western blotting and immunostaining. Bim was stably knocked down by short hairpin RNA. Immunoprecipitation was applied to detect the interaction between Bim and Bcl-2. Both subcutaneous and colorectal orthotopic tumor implantation models were used in nude mice to investigate the effect of PAO in vivo.

Results: PAO triggered Cyt c release and apoptosis in a Bax/Bak-independent manner. Bim and Bcl-2 were both involved in this process. PAO augmented the expression of Bim and strengthened the interaction between Bim and Bcl-2. Furthermore, PAO attenuated the growth of Bax-deficient cancer cells in vivo.

Conclusions: Our results showed that PAO induced apoptosis in chemotherapy-resistant cancer cells, which suggests that PAO has the potential to serve as a chemotherapeutic agent for Bax- and Bak-deficient cancers.

Introduction

Most anticancer drugs exert their effects through the induction of intrinsic apoptosis via mitochondrial pathway characterized by the release of cytochrome c (Cyt c) and caspase activation (1). Bcl-2 and its relatives play a pivotal role in determining the apoptotic process. Members of Bcl-2 family are divided into 3 subsets: antiapoptotic (Bcl-2, Bcl-xl, Bcl-w, Bfl1/A-1, Bcl-B, and Mcl-1) proteins, proapoptotic proteins (Bax and Bak), and BH3-only proteins (Bid, Bim, Bik, Bad, Noxa, and Puma). Overwhelming evidence has shown that Bax and Bak are indispensable for mediating Cyt c release from mitochondria during apoptosis. Cells without Bax or Bak are largely resistant to apoptotic agents and drugs. Mouse embryonic fibroblasts (MEF) isolated from Bax/Bak double knockout (DKO) mice are resistant to multiple death stimuli, including DNA-damaging agents, signal transduction through death receptors, growth factor deprivation, and endoplasmic reticulum stress (2–4). On the other hand, several studies including ours suggest the existence of a novel mechanism of intrinsic apoptosis in a Bax/Bak-independent pathway, although the mechanism remains elusive (5–7). BH3-only proteins are suggested to act as proapoptotic molecules which either directly interact with and activate Bax/Bak for their membrane permeabilizing activity or bind to antiapoptotic proteins to suppress their inhibition on Bax/Bak (2, 8). Alternatively, a unified model suggests that the BH3-only proteins induced conformational alterations and oligomerization of both Bax/Bak and Bcl-2/Bcl-xl (9). The conformation-activated Bcl-2/Bcl-xl have the potential to form pores in mitochondrial outer membrane (MOM), but the pore size is not large enough to mediate Cyt c release (10, 11). Functional conversion of Bcl-2 to a proapoptotic protein has been observed in other circumstances. Upon binding to Nur77 protein or peptide, Bcl-2 undergoes

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance
It has been well known that both bax and bak genes are frequent targets for mutation in a subset of human tumors. Loss or inactivation of Bax or Bak is considered as a mechanism of protection of cancer cells against apoptosis, and these cancers are largely resistant to chemotherapy. We showed in this study that phenylarsine oxide (PAO) induced apoptosis in Bax-deficient colorectal cancer cells which are drug resistant. PAO also attenuated the growth of these cells in subcutaneous and orthotopic xenograft tumors without significant concomitant adverse effects on nude mice. These all indicate that PAO has potential value for development in the therapy of Bax/Bak inactivation cancers. Bim and Bcl-2 were both involved in this process, which provides new targets in overcoming drug resistance during cancer therapy.

Materials and Methods

Reagents
PAO and ATO were purchased from Sigma. Antibodies (Abs) used for actin, Bim, Bcl-2, Bad, Puma, Bid, Bik, Mcl-1, Cyt c, PARP, and Bcl-xl were purchased from BD. Secondary Abs were purchased from Pharmingen. Annexin V kit of was obtained from Pharmingen. CaspACE In Situ kit was purchased from Promega. MitoTracker Red was purchased from Invitrogen. All other chemicals were purchased from Sigma unless otherwise specified.

Cell culture
SV40 T antigen–immortalized Bax/Bak DKO mouse embryonic fibroblasts (MEF), wild-type MEFs, bim−/− MEFs, and HCT116 bax−/− cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified 5% CO2 atmosphere at 37°C.

Apoptosis analysis by Hoechst, TUNEL, or Annexin V/propidium iodide staining
For Hoechst staining, cells were grown on glass cover slides at a density of 5 × 10^4 cells/mL in a 6-well plate. After treatments, cells were stained with Hoechst in PBS for 15 minutes at room temperature in the dark. Cells were then washed 3 times with PBS and analyzed with a fluorescence microscope. At least 200 cells were counted.

For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, cells were grown on glass coverslips at a density of 5 × 10^4 cells/mL in a six-well plate. After treatments, cells were stained by the Cell Death Detection Kit according to manufacturer’s instructions. Apoptotic cells were visualized under fluorescence microscopy.

Quantitative apoptosis was detected by flow cytometric analysis after staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) by a commercially available kit according to manufacturer’s instructions. All data were analyzed with Cell Quest software (BD).

Detection of caspase activity
Cells were treated as indicated, then collected and washed with PBS. CaspACE In Situ Marker was added to the cells to a final concentration of 10 μmol/L and incubated for 20 minutes in the dark. Cells were then washed 3 times with PBS and resuspended in 400 μL PBS and analyzed with a FACScan.

Cell fractionation assay
Cells were homogenized with a Dounce homogenizer, and the homogenate was centrifuged at 1,000 × g for 5 minutes to remove unbroken cells and nuclei. The cytosolic and mitochondrial fractions were obtained by further centrifugation as follows. Collect and spin the supernatant at 10,000 × g for 15 minutes. Collect the
pellet which contains heavy membrane including mitochondria. Collect supernatant and spin at 100,000 \( \times g \) for 1 hour to obtain cytosolic fraction and light membrane fraction.

**Immunostaining assay**

The cells were grown on glass cover slips, treated with PAO and stained with 50 nmol/L MitoTracker Red for 30 minutes at 37°C. After washed with PBS, fixed in 3.7% formaldehyde/PBS solution, the cells were incubated in 0.1% Triton X-100/PBS to be permeabilized. Primary Abs were diluted in 1% bovine serum albumin (BSA)/PBS and incubated with the cells at 4°C for 12 hours. The FITC-conjugated secondary Abs were diluted in 1% BSA/PBS and incubated at room temperature for 2 hours. The cells were examined by confocal microscopy using the Zeiss LSM 510 META.

**Reverse transcriptase PCR**

Total RNA was isolated from the cells with PAO treatment or not for reverse transcription and generation of cDNA. Primers for all the 3 isoforms of mouse Bim were designed...
Subcutaneous tumor implantation model

The DKO MEFs were transformed with KRasV12 and E1A. DKO MEFs were cultured in medium containing 1.5 µmol/L PAO –+ + + –. Five µmol/L ATO ––––+. The mice were euthanized after PAO treatment for 2 weeks. Tumor size was monitored by measurement of the length (a) and width (b) with a slide gauge. Tumor volumes (V) were calculated according to the formula: V = a × b²/2. Tumors were excised and stained by TUNEL staining. Apoptotic cells were visualized under fluorescence microscopy.

Orthotopic tumor implantation model

Four- to 5-week-old nude nu/nu mice were anaesthetized with phenobarbital during the surgical procedure. The caecum was exteriorized through a small midline laparotomy and 3 × 10⁶ HCT116 bax⁻/⁻ cells in 100-µL PBS, respectively, were injected into the oxter of 4 to 5 weeks old nude nu/nu mice with a 27-gauge needle. Two groups of mice (5 animals each group) were used and maintained in specific pathogen-free (SPF) conditions. One week after implantation, animals were intraperitoneally treated with PAO (1 mg/kg/2 days) or vehicle control [dimethyl sulfoxide (DMSO)], respectively. The mice were euthanized after PAO treatment for 2 weeks. Tumor size was monitored by measurement of the length (a) and width (b) with a slide gauge. Tumor volumes (V) were calculated according to the formula: V = a × b²/2. Tumors were excised and stained by TUNEL staining. Apoptotic cells were visualized under fluorescence microscopy.

Stable knockdown of Bim

The mouse Bim short hairpin RNA (shRNA) sequence (TGATGTAAGTTCTGAGTGTG) was inserted into pSilencer 2.1-CMV-Hygro cut by BamHI and HindIII. Control scrambled and shRNA plasmids (1.5 mg) were transfected into DKO MEFs plated at 50% confluence in six-well plates using Lipofectamine 2000 (Invitrogen). Transfectants were selected in culture medium containing 100 µg/mL hygromycin to enrich the culture for cells that were successfully transfected.

Immunoprecipitation for detecting Bim and Bcl-2 interaction

Cells were lysed with 1% NP-40 lysis buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 1% NP-40, pH 7.4) containing protease inhibitors. Total protein (500 µg) was incubated with 2 µg of anti-Bim antibody (Ab) in 500 µL of NP-40 lysis buffer at 4°C overnight on a rotator. Immunoprecipitates were collected by incubating with 20 µL protein A agarose for 2 hours at 4°C, followed by centrifugation for 1 minute. The pellets were washed 3 times with NP-40 lysis buffer, and beads were boiled in loading buffer and analyzed by Western blotting using the anti-Bcl-2 Ab.

Subcutaneous tumor implantation model

The DKO MEFs were transformed with KRasV12 and E1A. The cells were harvested by centrifugation and suspended in Hank’s balanced salt solution (HBSS). A tumor cell suspension (5 × 10⁶ KRasV12/E1A DKO MEFs or 3 × 10⁶ HCT116 bax⁻/⁻ cells in 100-µL PBS, respectively) was injected into the oxter of 4 to 5 weeks old nude nu/nu mice with a 27-gauge needle. Two groups of mice (5 animals each group) were used and maintained in specific pathogen-free (SPF) conditions. One week after implantation, animals were intraperitoneally treated with PAO (1 mg/kg/2 days) or vehicle control [dimethyl sulfoxide (DMSO)], respectively. The mice were euthanized after PAO treatment for 2 weeks. Primary tumors in the cecum were excised, the final tumor volumes (V) were calculated according to the formula: V = a × b²/2. (a, length; b,
width), and the number of metastasis colonies was counted in the intestines. Each tumor tissue excised from mice was snap frozen in liquid nitrogen and divided into 2 parts. The first part was fixed in formalin and embedded in paraffin. Serial sections of the intestine were prepared and stained by hematoxylin and eosin (H&E) staining for histopathologic evaluation. The second part was stained by TUNEL staining. Apoptotic cells were visualized under fluorescence microscopy. All protocols were approved by the Institutional Animal Care and Use Committee of Institute of Zoology accredited by AAALAC International.

Statistical analysis
Data are expressed as mean ± SEM. Significant differences between values under different experimental conditions were determined by paired or unpaired Student t test and ANOVA with repeated measurements, and P < 0.05 was considered statistically significant.

Results
PAO induces intrinsic apoptosis in Bax- and Bak-deficient cells
We screened a number of compounds for their capacity to induce cell death in SV40 T antigen–immortalized DKO MEFs by MTT assay and found that PAO induced a significant reduction of viability in these cells (data not shown). However, other agents or drugs including ATO had no effect on the viability of DKO MEFs. To further examine PAO-induced apoptosis, we used Annexin V/PI double staining assay. The results showed that the increase of Annexin V–positive population was in both time- and concentration-dependent manners after PAO treatment (Fig. 1A). The typical apoptotic hallmark, the exposure of phosphatidylserine on cell surface, was observed after 1.5 μmol/L PAO treatment for 24 hours (Fig. 1A).

To show that PAO could induce typical intrinsic apoptotic cell death in DKO MEFs, we analyzed intracellular

![Image](image-url)
caspase activity with CaspACE In Situ Marker. Caspase was activated by PAO both in time- and concentration-dependent manners (Fig. 1B). PAO also induced the proteolytic cleavage of PARP which is the substrate of caspase-3 in DKO MEFs (Fig. 1C). The caspase inhibitor (z-VAD-fmk) inhibited PAO-induced phosphatidylserine exposure and caspase activation (data not shown). In addition, nucleus condensation and fragmentation, which is a defining characteristic for apoptosis, was detected by Hoechst staining after PAO treatment (Fig. 1D). Collectively, the results showed that PAO induced intrinsic apoptosis in Bax- and Bak-deficient cells.

Furthermore, we tested the effect of PAO on cancerous cells with HCT116 and HCT116 bax−/− cell lines, respectively. Dramatically, PAO rather than ATO induced apoptosis which was in a time-dependent manner in these colorectal cancer cells, regardless of Bax absence (Fig. 1E). Data of TUNEL staining (Fig. 1F) and caspase activation assay (Supplementary Fig. S1) also showed that PAO could induce apoptotic cell death in Bax-deficient cancer cells, which is resistant to multiple death stimuli.

PAO induces Cyt c release in a Bax/Bak-independent pathway

The release of Cyt c from mitochondria into cytosol is a defining event which triggers intrinsic mitochondrial apoptotic signals (23). Previous reports have suggested the existence of Cyt c release without Bax and Bak (5, 6). We thus studied Cyt c release during PAO-induced apoptosis in the absence of Bax and Bak. We first exposed DKO MEFs to 1.5 μmol/L PAO, then fractionated and extracted mitochondria to detect the protein levels of Cyt c in the cytosolic and mitochondrial fractions, respectively. The Western blotting data revealed that PAO induced Cyt c release in a time-dependent manner, as the amount of Cyt c increased in the cytosolic fraction with an accompanied decrease in the mitochondrial fraction (Fig. 2A). We also examined intracellular distribution of endogenous Cyt c by immunostaining. Cyt c showed a diffused distribution in cytosol after 1.5 μmol/L PAO treatment for 12 hours, whereas it was well colocalized with mitochondria in untreated cells (Fig. 2B). Consistent with the fractionation data, these results indicate that PAO induced Cyt c release in a Bax/Bak-independent manner. Cyt c release was also detected in Bax-deficient colorectal cancer cells (Fig. 2C), which further indicates that PAO-induced apoptosis is through a mitochondrial signaling pathway and PAO-induced Cyt c release is Bax independent.

Bim is upregulated in Bax/Bak-independent apoptosis

Next, we sought to understand the mechanism of PAO-induced Bax/Bak-independent apoptosis. Despite the absence of Bax and Bak, other Bcl-2 family members could
be activated to mediate Cyt c release as we previously suggested (7). We thus tested the expressions of several members of Bcl-2 family (Puma, Bik, Bim, Bid, Bad, Bcl-2, Bcl-xL, and Mcl-1) during PAO-induced apoptosis. Interestingly, we found the protein expressions of 3 isoforms of Bim including BimEL, BimL, and BimS were all upregulated in DKO MEFs after the treatment with 1.5 μmol/L PAO for 6 hours, whereas the expressions of other Bcl-2 family proteins had no significant changes upon PAO treatment (Fig. 3A). Similarly, the protein expressions of all 3 isoforms of Bim were upregulated in HCT116 bax−/− cells after PAO treatment for 12 hours (Fig. 3C). We further analyzed mRNA expression of Bim by reverse transcriptase PCR (RT-PCR) and found that the amount of mRNA of these isoforms was increased after PAO treatment (Supplementary Fig. S2A). More interestingly, fractionation followed by Western blotting analysis showed the protein levels of these isoforms of Bim were increased both in cytosolic and mitochondrial fractions during PAO-induced apoptosis (Fig. 3B). Immunostaining analysis by confocal microscopy also showed that the expression of endogenous Bim was induced both in cytosol and in mitochondria in the cells treated by PAO, compared with control ones (Supplementary Fig. S2B).

**Bim and Bcl-2 are both involved in Bax/Bak-independent apoptosis**

Bim has been characterized as serving as a death ligand, which regulates other members of the Bcl-2 family as a proapoptotic molecule once it has been activated (2). To find out whether the upregulation of Bim induced by PAO is required for apoptosis in DKO MEFs, we knocked down the expression of Bim. A shRNA which is specific to all the 3 isoforms of Bim was stably transfected into the DKO MEFs (Fig. 4A). As we expected, knockdown of Bim significantly inhibited PAO-induced apoptosis in DKO MEFs, compared with those stably transfected with scrambled shRNA (Fig. 4B). To further show the role of Bim in PAO-induced apoptosis, we treated bim−/− MEFs and the wild-type MEFs with PAO, respectively. Annexin V/PI double staining assay showed that PAO-induced apoptotic cells were decreased significantly in the absence of Bim, regardless of the presence of Bax/Bak (Fig. 4C). All these data revealed that Bim was involved in and initiated PAO-induced apoptosis in DKO MEFs.

It has been reported that Bim could suppress Bcl-2 which is a primary inhibitor of apoptosis and constitutively localizes on outer mitochondrial membrane (2). As shown in Fig. 3B, the protein expression of Bim on mitochondria

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**Figure 4.** Bim is involved in PAO-induced apoptosis. A, the efficiency of Bim knockdown was confirmed by Western blotting. B, stable Bim knockdown (shRNA) or scrambled (SC) DKO MEFs were treated with 1.5 μmol/L PAO for 30 hours, respectively. The percentage of apoptotic cells was detected by Annexin V/PI staining of flow cytometric analysis. Data are mean ± SEM from 3 independent experiments. ***, P < 0.001 versus scramble. C, wild-type (WT) or bim−/− MEFs were treated with 1.5 μmol/L PAO for the indicated times, respectively. The percentage of apoptotic cells was detected by Annexin V/PI staining of flow cytometric analysis. Data are mean ± SEM from 3 independent experiments. ***, P < 0.001 versus wild-type.
PAO Induces Bax/Bak-Independent Apoptosis

Figure 4. (Continued) D, PAO enhances the interaction between Bim and Bcl-2. DKO MEFs were exposed to 1.5 μmol/L PAO for the indicated times, harvested, lysed in NP-40 lysis buffer, and immunoprecipitated with Bim Ab. Immunoprecipitates (IP) were subjected to Western blotting using Bcl-2 Ab. The whole-cell lysate was analyzed by Western blotting to confirm the expression of Bcl-2. β-Actin was used as a loading control. E, YC137 enhances PAO-induced apoptosis. After pretreatment with 1 μmol/L YC137 for 1 hour or not, DKO MEFs were incubated with 1.5 μmol/L PAO for 12 hours. The percentage of apoptotic cells was detected by Annexin V/PI staining of flow cytometric analysis. Data are mean ± SEM from 3 independent experiments. ***P < 0.001 versus vehicle control.

was increased after PAO treatment, and immunostaining data confirmed the localization of Bim on mitochondria (Supplementary Fig. S2B). We thus supposed that PAO induced apoptosis in Bax- and Bak-deficient cells by augmenting the expression of Bim on mitochondria where it inhibited Bcl-2. To test this hypothesis, we detected the interaction between endogenous Bim and Bcl-2 by immunoprecipitation. The amount of Bim/Bcl-2 complex was increased after PAO treatment for 6 hours, indicating that Bim interacted with Bcl-2 and the interaction between them was strengthened during PAO-induced apoptosis (Fig. 4D). To further investigate the role of Bcl-2 in Bax/Bak-independent apoptosis, we used YC137 as a specific inhibitor to Bcl-2 (24). PAO-induced apoptotic cells were significantly increased in YC137-pretreated DKO MEFs, compared with control ones in the absence of YC137 (Fig. 4E). This Bcl-2 inhibitor augmented PAO-induced apoptosis, suggesting that Bcl-2 was involved in this process.

PAO attenuates the growth of DKO MEFs in vivo

We next tested the effects of PAO on the growth of xenograft tumors in vivo. The immunodeficient nude mice were implanted with KRasV12/E1A-transformed DKO MEFs in the oxtor and underwent intraperitoneal treatment with PAO (1 mg/kg/2 days) or DMSO (the vehicle control) one week later. The tumor volume was measured every other day, and it was found that PAO inhibited tumor growth (Fig. 5A). Two weeks after PAO treatments, the mice were euthanized, and the xenograft tumors were isolated. The average tumor weight of the treated group was significantly reduced, compared with that of the control group (Fig. 5B and C). Importantly, this dose of PAO inhibited tumor growth in the mice without significant converse effects on body weight (Fig. 5D). Moreover, we detected PAO-induced apoptosis in vivo in tumors formed with DKO MEFs by TUNEL assay in situ. Fluorescence microscopy images showed an increase of apoptotic cells in tumors from the group treated with PAO, compared with those from control group (Fig. 5E).

PAO attenuates the growth of Bax-deficient cancer cells

In the orthotopic tumor model, the treatment started one week after HCT116 bax−/− cells implantation and was continued for 2 weeks. The mice were euthanized, and the tumors were isolated, dissected, and measured. The results showed that PAO decreased orthotopic tumor growth (Fig. 6E) and the tumor volume in PAO treatment group was significantly lower than that in the control group (Fig. 6F). Furthermore, the presence of orthotopic xenograft tumors in the cecum was confirmed by H&E staining (Fig. 6H).
PAO-induced apoptotic cell death was confirmed by TUNEL staining (Fig. 6I), and PAO-induced apoptosis was not detected with TUNEL assay in normal colorectal tissues (Supplementary Fig. S3).

Discussion

In this study, we found that PAO could induce typical intrinsic apoptosis in Bax/Bak DKO MEFs, which are largely resistant to multiple chemotherapeutical drugs and apoptosis-inducing agents. PAO induced dramatic increase of Bim expression which is involved in Bax/Bak-independent apoptosis. PAO also induced apoptotic cell death in chemotherapy-resistant colorectal cancer cells, such as HCT116 bax"/" cells. The results contrast with our previous report which showed that ATO induced apoptosis in a Bax-dependent manner (22). Our data also showed that Bim was not essential for ATO-induced apoptosis in MEFs as ATO could induce apoptosis in the absence of Bim (Supplementary Fig. S6D), although Bim was reported to be transcriptional upregulated and activated by ATO in neuronal cell line (25, 26). Clearly, PAO is much more powerful in overcoming drug resistant cancers that are refractory to conventional chemotherapeutical agents. Our data suggested that PAO treatment had advantages over the conventional anticancer therapies that rely on the Bax or Bak to kill cancer cells. Moreover, the orthotopic tumor implantation model in nude mice clearly showed that PAO inhibited the growth of Bax-deficient cancer cells in vivo without significant side effects on mice. It has been well known that both bax and bak genes are frequent targets for mutation in a subset of human tumors, including hematopoietic malignancies, breast cancers, gastric cancers, colorectal cancers, and prostate cancers (27–31). Loss or mutational inactivation of Bax or Bak is often reported and considered as a mechanism of protection against apoptosis in cancer cells, and these cancers are largely resistant to chemotherapy, including arsenic compounds treatments (32–35). Therefore, our study implicates that PAO has potential value for overcoming chemotherapy resistance in the treatments of cancers, especially in the treatments of Bax or Bak inactivation cancers.

Our results provide new evidence for the existence of a Bax/Bak-independent apoptosis pathway to mediate Cyt c release. It is recently reported that apoptosis could be induced through a mitochondrial permeability transition pore (mPTP)—not Bax/Bak-dependent manner. However, here, specific mPTP inhibitor cyclosporine A (CsA) or bongkrekic acid (BA) fails to inhibit PAO-induced apoptosis (Supplementary Figs. S4 and S6C). Prior studies showed that PAO increased the concentration of intracellular calcium (20), which was indicated to be involved in Bax/Bak-independent mechanism of Cyt c release (5). Here, we found that EGTA-am had no effect on PAO-induced apoptosis (Supplementary Fig. S5), suggesting that intracellular calcium is not involved in this process. Ionomycin could indeed induce cell death in HCT116 bax"/" cells in an EGTA-am–inhibitable manner. However, activated caspase and other typical intrinsic apoptotic hallmarks were not detected in ionomycin-induced cell death (data not shown). Intriguingly, we showed that PAO upregulated the expression of Bim which is involved in PAO-induced apoptosis in the absence of Bax/Bak. PAO strengthened the...
interaction between Bim and Bcl-2. Moreover, specific suppression of Bcl-2 by YC137 can enhance PAO-induced apoptosis. This is in line with our previous finding that Bcl-2 underwent structural change during Bax/Bak-independent apoptosis, which might convert the protective molecule into a killer (7). Our recent results showed that Bim binding to Bcl-2 could trigger conformational and functional conversion of Bcl-2, which permeabilized the MOM, thereby triggering Cyt c release during the apoptosis process in the absence of Bax and Bak. The exact mechanism of how Bim interacts with Bcl-2 for the proapoptotic action is still a subject in future study.

Figure 6. PAO attenuates the growth of Bax-deficient cancer cells in vivo. A, tumor volume curves. One week after the injection of HCT116 bax−/− cells into the oxters, the mice underwent intraperitoneal treatment with vehicle control (DMSO) or PAO (1 mg/kg/2 days), respectively, for 2 weeks (n = 5). B, representative images of isolated tumors. Two weeks after PAO treatment, the mice were euthanized and the xenograft tumors were isolated. Scale bar represents 1 cm. C, the tumor weight of mice at 14 days after treatment. *, P < 0.05 versus vehicle control. D, average body weight of mice at 14 days after treatment. NS, no statistical significance. E, TUNEL staining show apoptotic cells in tumors in situ. Scale bar represents 100 μm. F, representative necropsy photographs of mice with orthotopic xenograft tumors (arrows). One week after orthotopically implantation of HCT116 bax−/− cells into the ceca, the mice underwent intraperitoneal treatment with vehicle control (DMSO) or PAO (1 mg/kg/2 days), respectively, for 2 weeks. Scale bar represents 1 cm. G, average tumor volume of mice at 14 days after treatment. *, P < 0.05 versus vehicle control (n = 5). H, H&E staining of intestinal tumors. N, normal tissue; T, tumor. The poorly differentiated morphology of tumor cells is typical of high-grade adenocarcinomas. Scale bar represents 100 μm. I, TUNEL staining show apoptotic cells in orthotopic tumors. Scale bar represents 100 μm.
Nevertheless, the unexpected effects of Bim and Bcl-2 on PAO-induced apoptosis provide a novel view to explore the mechanism of Bax/Bak-independent apoptosis and imply new targets in the treatments of Bax or Bak inactivation cancers which are chemoresistance therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Q. Ma and Q. Chen designed the experiments. B. Ni, Q. Ma, B. Li, L. Zhao, Y. Liu, and Y. Zhu carried out the experiments. Q. Ma made the data statistics and analysis. Q. Ma and Q. Chen drafted the manuscript. All authors read and approved the final manuscript.

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