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Arsenic Trioxide Inhibits Cholangiocarcinoma Cell Growth and induces Apoptosis

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Abstract Arsenic trioxide (As_2O_3), an ingredient in many traditional Chinese medicines, has drawn broad attention due to its therapeutic effects on a variety of cancers, including some solid tumors. However, the effects of As_2O_3 on cholangiocarcinoma have not been reported. In the present study, we demonstrate for the first time that clinically obtainable concentrations of As_2O_3 inhibit cell growth and induce apoptosis in human cholangiocarcinoma SK-ChA-1 cells. As_2O_3-induced apoptosis was partially inhibited by caspase inhibitor and accompanied by changes in the expression of Bcl-2 family proteins, decrease of mitochondrial membrane potential (MMP), release of cytochrome C from mitochondria, activation of caspase-3, caspase-9, and cleavage of poly (ADP-ribose) polymerase (PARP). Thus As_2O_3 induces apoptosis in SK-ChA-1 cells via mitochondria-mediated, caspases-dependent pathways. As_2O_3 inhibition of Akt phosphorylation may contribute to As_2O_3-mediated cholangiocarcinoma cell growth inhibition and apoptosis induction.

Keywords Arsenic trioxide · Apoptosis · Cholangiocarcinoma · Mitochondrial pathway · AKT

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Introduction

Cholangiocarcinoma is the second most common type of primary hepatic tumor, with increasing incidence and high mortality [1,2]. The disease is notoriously difficult to diagnose and is frequently fatal due to its late clinical presentation and the lack of effective non-surgical therapeutic modalities [3]. Chemotherapy has not been shown to substantially improve survival in patients with resected or unresected cholangiocarcinoma [4]. The overall 5 year survival rate for patients with cholangiocarcinoma, including patients that have undergone resection, is less than 5% and has not changed significantly over the past 30 years [5]. Thus novel therapeutic strategies are needed for cholangiocarcinoma which exploit potentially effective medicinal agents to improve clinical outcome.

For centuries, arsenic trioxide (As_2O_3) has been a major ingredient in traditional Chinese medicines used to treat several human diseases, including anemia, dyspepsia, and some types of tumors [6]. As_2O_3 has drawn broad attention in the past decade due to its dramatic therapeutic effect on acute promyelocytic leukemia (APL) [7,8]. Currently, As_2O_3 is indicated as a broad-spectrum anticancer medicine

used for treatment of a variety of cancers, including esophageal, prostate, ovarian, hepatocellular, lung, and gastric carcinomas [9–13]. However, to our knowledge, there have been no reports on the effects of As_2O_3 on cholangiocarcinoma. In the present study, we investigated the cytotoxic and apoptotic effects of As_2O_3 on cholangiocarcinoma SK-ChA-1 cells and the possible underlying molecular mechanisms of these effects.

Materials and Methods

Reagents As_2O_3, rhodamine 123 (Rh123), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Hoechst 33342 were purchased from Sigma (St. Louis, Mo USA). RPMI1640 medium and heat-inactivated fetal calf serum were from (Carlsbad, CA USA). The XTT proliferation kit was purchased from Boehringer Mannheim (Mannheim, Germany). The annexin V-FITC apoptosis detection kit I was purchased from BD Biosciences Pharmingen (San Jose, CA USA). Caspase activity Colorimetric Assay Kit was purchased from Assay Designs (Ann Arbor, MI USA). Antibodies used were: anti-cleaved PARP (Promega, Madison, WI); anti-cytochrome C (Pharmingen); and anti-Bax, anti-Bcl-2, anti-Bad, anti-Bid, anti-Bcl-xL, anti-AKT Ab, anti-phospho–AKT (Thr308), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA USA). Anti-mouse, anti-rabbit and anti-goat IgG-HRP (Santa Cruz) were used as secondary antibodies.

Cell lines and Cell Culture Human cholangiocarcinoma cells (SK-ChA-1) were provided by the central laboratory of Sun Yat-sen University Cancer Centre. Cells were grown in RPMI1640 medium supplemented with 2 mmol/L L-glutamine, penicillin (5 U/ml), streptomycin (5 μg/ml), and 10% heat-inactivated fetal calf serum in a humidified incubator in a 5% CO_2 atmosphere at 37°C. The culture media was replenished every 2–3 days and cell passage was performed twice a week in order to maintain the cell viability and exponential cell growth.

Cell Viability Assay Cell viability was measured using the XTT assay, which is based on the reduction of tetrazolium salt to soluble formazan compounds by mitochondrial enzymes. Cells (1×10^4 cells/well) were seeded in a 96-well plate in 100 μL of RPMI 1640 medium. After incubation at 37°C for 24 h, cells were exposed to various concentrations (0, 0.5, 1, 2, 3, and 4 μmol/L) of As_2O_3 and to 2 μmol/L As_2O_3 in serum-free RPMI1640 medium for different amounts of time. After incubation with As_2O_3, 50 μL of XTT/PMS mixture (50 μmol/L PMS, 0.1% XTT in medium) were added to the cell culture media. After incubation at 37°C for 3 h, the absorbance at 450 nm of the samples was measured in an enzyme-linked immunosorbent assay (ELISA) reader. Cell viability was calculated as a percentage of absorbance of XTT in individual As_2O_3-treated wells versus untreated control wells.

Morphological Changes Apoptotic cells were identified on the basis of nuclear morphology changes, such as chromatin condensation and DNA fragmentation. Nuclear staining with Hoechst 33342 and propidium iodide (PI) was performed as previously described [14]. Briefly, cells (1×10^5 cells/well) were seeded in a 12-well plate and treated with As_2O_3. The treated cell culture was further incubated with 5 μg/ml Hoechst 33342, and subsequently with 5 μg/ml PI at 37°C in the dark for 5 min. Blue (Hoechst 33342) or red (PI) fluorescence was examined by fluorescence microscopy (Nikon TE 2000-U, Japan).

Flow Cytometric Apoptosis Analysis SK-ChA-1 cell apoptosis was assessed using the annexin V-FITC apoptosis detection kit I according to the manufacturer's protocol. Briefly, the cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. 1×10^5 cells (100 μL of the solution) were transferred to a 5 ml culture tube, and then 5 μL of Annexin V-FITC and 5 μL of PI were added to the tube. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Following incubation, 400 μL of 1× binding buffer was added to each tube and the samples were immediately analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ USA). In the caspase inhibitor assay, cells were treated with 0–4 μmol/L As_2O_3 for 24 h, with or without 100 μM z-VAD-fmk pretreatment for 2 h, and then apoptosis was determined by annexin V/PI dual staining apoptosis assay as described above.

Caspase Assays Caspase-3, -8, and -9 activity was measured using colorimetric assay kits (Assay Designs) according to the manufacturer's instructions. Briefly, SK-ChA-1 cells treated with indicated concentrations of As_2O_3 for different period of time. After treatment, cells were collected and lysed with a chilled lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM EDTA, 0.2% Triotn X-100, 1 mM PMSF, 10 μg/ml aprotinin and 5 mM DTT) for 10 min on ice. The lysates were centrifuged for 10 min at 10000 g at 4°C. The supernatant containing 100 μg of protein was incubated with 0.2 mM of DEVD-pNA (caspase-3 substrate), IETD-pNA (caspase-8 substrate), or LEHD-pNA (caspase-9 substrate). Samples were read at 405 nm in a microplate reader (Bio-Tek Instruments) and

expressed as fold increase on the basal level (DMSO-treated cells).

Western Blot Analysis Cells were cultured with the indicated concentrations of As_2O_3 for the specified times, harvested, washed, and lysed using RIPA buffer containing protease inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted on nitrocellulose membranes. Membranes were blocked for 1 h in a non-fat dried milk solution (5% in Tris-buffered saline) containing 0.5% Tween 20 and then incubated with the appropriate primary antibody at a 1:1000 dilution in buffer for 2 h at room temperature. After rinsing, blots were incubated with a HRP-coupled secondary antibody (1:2000 dilution in buffer) for 2 h at room temperature. Membrane-bound HRP-labeled protein bands were detected using enhanced chemiluminescence agents and X-ray film. The film was scanned and the bands were analyzed and quantified with GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA USA). The density of control was taken as 1 and results of treatment are expressed in relation to the control in terms of relative unit (RU).

Cytochrome c Release from Mitochondria Desired cells were washed with PBS, and resuspended in isotonic homogenizing buffer (250 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 10 mM HEPES-KOH, pH 7.4). After 30 min incubation at 4°C, cells were homogenized with a glass Dounce homogenizer (30 strokes) and centrifuged at 700*g* for 10 min. The resulting supernatant was collected as the cytosolic fraction and protein concentrations were measured using a DC protein assay kit. Twenty μg of protein were analyzed by Western blot analysis for released cytochrome c.

Measurement of Mitochondrial Membrane Potential Mitochondrial membrane potential (MMP) was measured according to the previously reported methods with some modifications [15]. Briefly, after treatment, cells were collected and incubated with medium containing Rh123 (10 μg/ml) for 30 min and analyzed by flow cytometry. The decoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control to induce membrane depolarization. In all cases, samples were gated to exclude cellular debris.

Statistical Analysis Data are expressed as the mean ± standard deviation (SD) of triplicate experiments. The statistically significant differences between samples were determined using the Student's *t*-test. Differences of $p<0.01$ or $p < 0.001$ were considered significantly different.

Results

As_2O_3 Inhibits the Growth of SK-ChA-1 Cells and Induces Apoptosis

An As_2O_3 dose-response experiment was performed in order to define an effective concentration for inhibition of SK-ChA-1 cell growth. As shown in Fig. 1a, cell exposure to As_2O_3 for 24 h decreased the number of metabolically active cells in a concentration-dependent manner, with an IC_{50} of approximately 2 μmol/L. Using this IC_{50} concentration of 2 μmol/L, the number of viable cells was detected at 0, 6, 12, 24, 48, 72 h after exposure to As_2O_3. As shown in Fig. 1b, As_2O_3-induced cytotoxicity in SK-ChA-1 cells was time-dependent.

To determine whether As_2O_3-induced SK-ChA-1 cell cytotoxicity was caused by apoptosis, Hoechst/PI dual staining and flow cytometric assay were performed. After incubation with 2 μmol/L As_2O_3 for 24 h, SK-ChA-1 cells displayed typical apoptotic features of fragmented nuclei and chromatin condensation, some of which were positive to PI staining, suggesting apoptotic cell death (Fig. 2a). To quantify apoptosis, SK-ChA-1 cells incubated with As_2O_3 (0, 1, 2, 4 μmol/L) for 24 h were stained with Annexin V and PI and analyzed by flow cytometry. Although annexin V does not stain exclusively apoptotic cell [16], the morphological data support that apoptotic death is dominant in our case. Consequently,the criteria of annexin V binding on dying cells can be considered as specific for

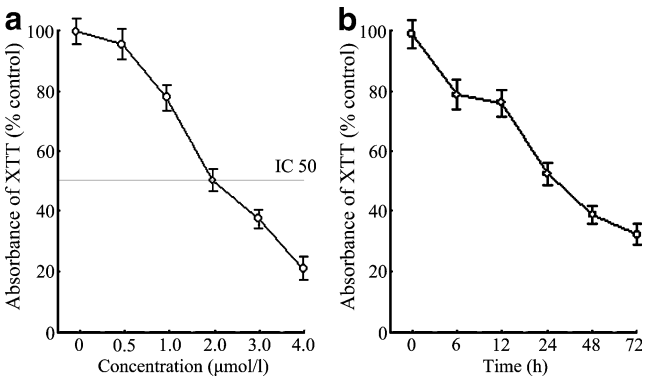

Fig. 1 As_2O_3 inhibited SK-ChA-1 cell growth. (**a**) SK-ChA-1 cells were treated with various concentrations of As_2O_3 for 24 h. (**b**) SK-ChA-1 cells were treated with 2 μmol/L As_2O_3 for different durations. Data are expressed as the mean ± SD of 3 replicate experiments

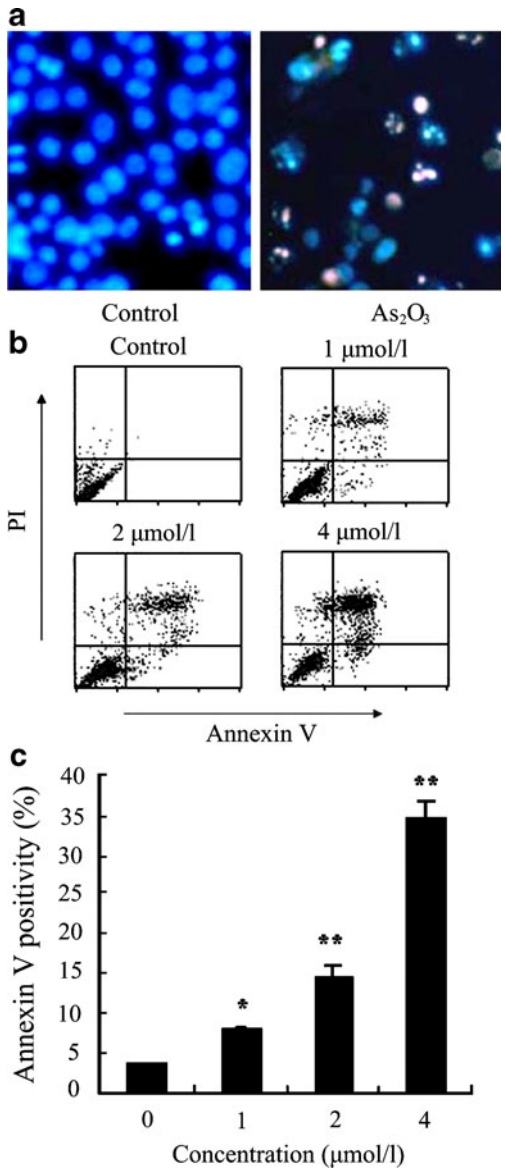

Fig. 2 As_2O_3 induces apoptosis in SK-ChA-1 cells. (**a**) Morphological changes of SK-ChA-1 cells induced by As_2O_3. Hoechst 33342/PI dual staining of nuclei from cells in the absence or presence of As_2O_3 (2 μmol/L) for 24 h. Viable cells had normal-shaped nuclei that are faintly stained with Hoechst 33342 (*right*). Apoptotic cells had shrunken nuclei with evidence of chromatin condensation, some of which were positive to PI staining (*left*). (**b**) Representative results of apoptosis quantification by dual Annexin V-FITC and propidium iodide (PI) staining and flow cytometry analysis of untreated cells or cells treated with various concentrations of As_2O_3 for 24 h. Similar results were obtained in replicate experiments. Based on the morphological observation, cells in the bottom right quadrants (FITC+ and PI-) and the upper right quadrants (FITC+ and PI+) represent early apoptotic and late apoptotic cells, respectively; bottom left quadrants (FITC- and PI-), living cells; upper left quadrants (FITC- and PI+), necrotic cells. (**c**) Apoptosis of SK-ChA-1 cells following exposure to increasing concentrations of As_2O_3 for 24 h. Results shown are the mean ± SD of 3 independent experiments. Significant differences from control experiments: $^{*}P<0.01$; $^{**}P<0.001$

apoptosis. As shown in Fig. 2b and c, the percentage of apoptotic cells increased in response to increasing doses of As_2O_3. These results are consistent with cell viability as determined by the XTT assay, suggesting that the inhibition of cell growth by As_2O_3 may be the result of induction of an apoptotic cell death pathway.

As_2O_3 Induces Caspase-dependent Apoptosis in SK-ChA-1 Cells

To determine whether As_2O_3-induced apoptosis was dependent upon caspase activation, SK-ChA-1 cells were cultured in the presence and absence of the broad-spectrum caspase inhibitor z-VAD-fmk and analyzed by annexin V/PI dual staining apoptosis assay. Pretreatment of SK-ChA-1 cells with 100 μM z-VAD-fmk resulted in inhibition of As_2O_3-induced apoptosis (Fig. 3a), indicating that its ability to induce cell death is z-VAD-fmk-inhibitable.

Considering that z-VAD-fmk-inhibitable cell death is not necessarily caspase-dependent [17], we further checked if caspase-3, the major effector caspase, and PARP, the main substrate of caspases, were involved in As_2O_3-induced apoptosis. The activation of caspase-3 was assayed by a caspase-3 colorimetric assay kit. Cleavage of PARP was analyzed by Western blot using an antibody for the 85 kDa cleaved PARP fragment. Colorimetric assay revealed that As_2O_3 treatment resulted in a marked increase in caspase-3 activity after 24 h in a dose-dependent manner (Fig. 3b). Correspondingly, the amount of cleaved PARP, as demonstrated by the appearance of the 85-kDa cleavage product, increased after As_2O_3 treatment of SK-ChA-1 cells in a dose-dependent manner (Fig. 3c). Caspase activity and PARP cleavage are intracellular signs of activation of the apoptotic machinery. Altogether, these data demonstrate that As_2O_3 induces cytotoxicity of SK-ChA-1 cells through an apoptotic mechanism dependent upon caspase activation.

As_2O_3 -induced Apoptosis is Mediated Through the Mitochondrial Pathway

To further characterize As_2O_3-induced apoptosis, we examined whether As_2O_3 activates the extrinsic or intrinsic apoptotic pathway in SK-ChA-1 cell. To determine which apoptotic pathway As_2O_3 activates, we examined the activity of caspase-8 and -9, the apical proteases in the extrinsic and intrinsic pathways, respectively, by colorimetric analysis. As shown in Fig. 4a, As_2O_3 treatment increased the activity of caspase-9 in a dose-dependent manner compared to vehicle-treated cells. Interestingly, caspase-8 activity in vehicle-treated and As_2O_3-treated cells remained unaffected. These results indicated that As_2O_3-

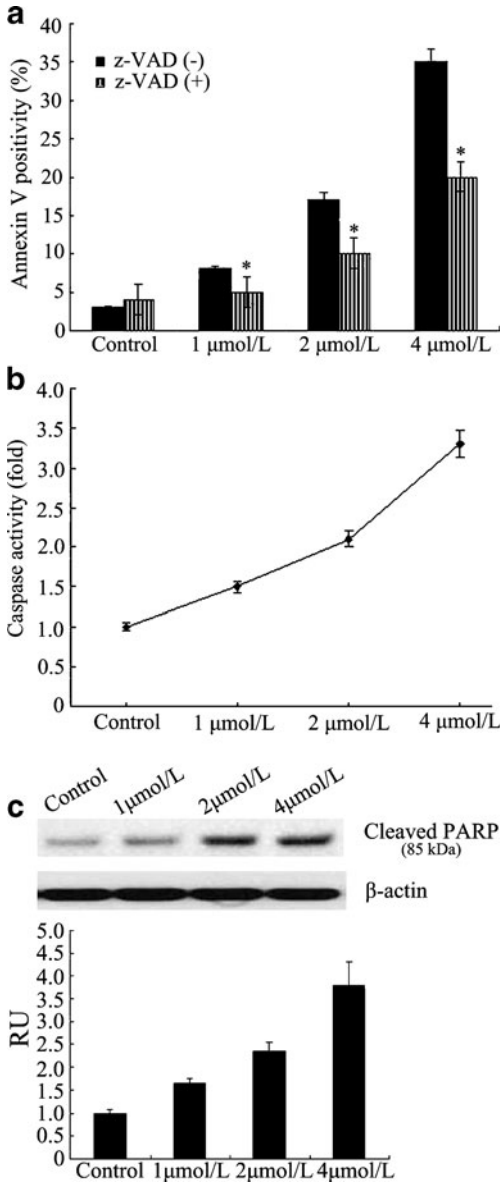

Fig. 3 As_2O_3 induces caspase-dependent apoptosis in SK-ChA-1 cells. (**a**) SK-ChA-1 cells were treated with 0-4 μmol/L As_2O_3 for 24 h, with or without 100 μM z-VAD-fmk pretreatment for 2 h, and then apoptosis was determined by annexin V/PI dual staining apoptosis assay. Data represent means ± SD of three independent experiments ($^*P<0.01$ when cells pretreated with z-VAD-fmk and As_2O_3 were compared with cells treated with As_2O_3 alone). (**b**) SK-ChA-1 cells were treated with 0-4 μmol/L As_2O_3 for 24 h, and then activation of caspase-3 was determined by colorimetric assay. Data represent means ± SD of three independent experiments. (**c**) SK-ChA-1 cells were treated with 0-4 μmol/L As_2O_3 for 24 h, and then whole-cell lysates were subjected to Western blotting to assess the expression of the 85 kDa cleaved PARP. *β*-actin was used as internal control to ensure that equal amounts of proteins were loaded in each lane. Results are representative of 3 replicate experiments (*upper*). The densities of the cleaved PARP bands were determined and the relative amount of the target protein was shown as RU (*lower*)

induced apoptosis was most likely to occur through the mitochondrial pathway.

To confirm whether mitochondrial events were involved in the induction of apoptosis, disruption of the MMP and release of mitochondrial cytochrome c in As_2O_3-treated cells were investigated by flow cytometry and immunoblotting. Flow-cytometric analysis revealed that exposure of SK-ChA-1 cells to As_2O_3 decreased the MMP in a dose-dependent manner (Fig. 4b), suggesting that mitochondria were involved in As_2O_3-induced apoptosis. Next the release of cytochrome c was investigated by Western blotting. As shown in Fig. 4c, the relative amount of cytochrome c in the cytosol of the cells treated with As_2O_3 was dramatically increased in a dose-dependent manner compared to the vehicle-treated cells.

To further examine whether important proapoptotic and antiapoptotic regulatory proteins could be modulated by As_2O_3 in SK-ChA-1 cells, we determined the expression of Bcl-2 family members by Western blot analysis. As shown in Fig. 4d, Bax and Bad were markedly up-regulated; Bcl-2 and Bcl-xL were down-regulated; Bak was unchanged after As_2O_3 treatment. Altogether, these findings demonstrate that As_2O_3-induced apoptosis in SK-ChA-1 cells is mediated through the mitochondrial pathway.

As_2O_3 Inhibits Akt Phosphorylation in SK-ChA-1 Cells

AKT, also referred to as PKB, is probably the best characterized kinase known to promote cellular survival [18]. To determine whether As_2O_3 affected the level of AKT phosphorylation, Akt phosphorylation status was examined by Western blot in As_2O_3-treated SK-ChA-1 cells. As shown in Fig. 5, As_2O_3 treatment lead to a marked reduction in phospho-Akt and a concomitant increase in unphosphorylated Akt, suggesting that As_2O_3 treatment inhibits Akt phosphorylation.

Discussion

This study demonstrates for the first time that As_2O_3 inhibits human cholangiocarcinoma SK-ChA-1 cell growth and survival in a dose- and time-dependent manner. The IC_{50} of As_2O_3 was 2 μmol/L in the cell line tested. Previous studies have shown that this concentration is clinically obtainable in serum and is well tolerated [19]. These results suggest that As_2O_3 may be a clinically useful chemotherapeutic agent in patients with cholangiocarcinoma.

To determine the mechanism of As_2O_3–induced inhibition of cell survival, we examined SK-ChA-1 cells treated

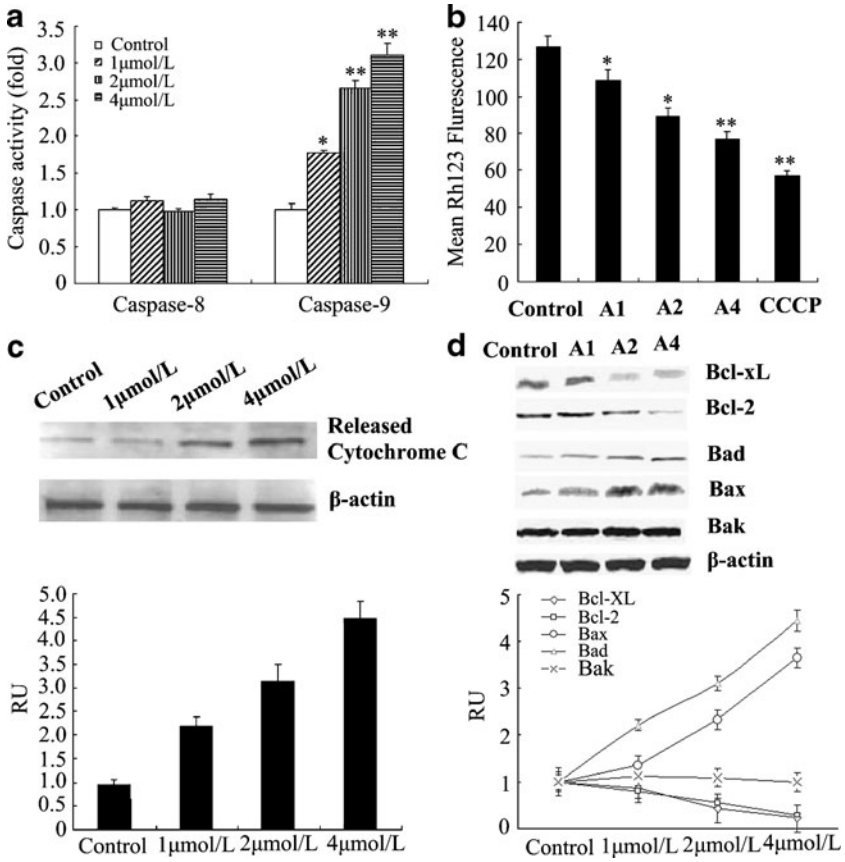

Fig. 4 As_2O_3-induced apoptosis is mediated through the mitochondrial pathway. (**a**) After treatment with As_2O_3 for 24 h, the cytosolic fraction of the cells was analyzed for changes in the activity of caspase-8, and -9 using colorimetric assay. Data represent means ± SD of three independent experiments (*p<0.01 or**p<0.001 versus control). (**b**) Effect of As_2O_3 on mitochondrial membrane potential in SK-ChA-1 cells. After treatment with 0-4 μmol/L As_2O_3 for 24 h, SK-ChA-1 cells were incubated with Rh123 (10 μg/ml) for 30 min, and then immediately subjected to flow-cytometric analysis. Cells treated with the mitochondrial uncoupler CCCP (10 μM) for 2 h were used for positive control. Results were expressed as mean Rh123 fluorescence (means ± SD of three independent experiments, (*p<0.01 or**p<0.001 versus control). A1, As_2O_3 1 μmol/L; A2, As_2O_3 2 μmol/L; A4, As_2O_3 4 μmol/L. (**c**) Determination of cytochrome c release. After treatment with As_2O_3 for 24 h, cytosolic fraction was isolated and the content of cytochrome c was examined by Western blot analysis. β-Actin was used as internal control to ensure that equal amounts of proteins were loaded in each lane. Results are representative of 3 replicate experiments (*upper*). The densities of the cytochrome c bands were determined and the relative amount of the target protein was shown as RU (*lower*). (**d**) Effect of As_2O_3 on expression of Bcl-2 family proteins. After treatment with 0–4 μmol/L As_2O_3 for 24 h, whole-cell lysates were subjected toWestern blotting to assess the expression of Bcl-2 family proteins. β-Actin was used as internal control to ensure that equal amounts of proteins were loaded in each lane. Results are representative of 3 replicate experiments (*upper*). A1, As_2O_3 1 μmol/L; A2, As_2O_3 2 μmol/L; A4, As_2O_3 4 μmol/L. The densities of the Bcl-2 family proteins bands were determined and the relative amount of the target protein was shown as RU (*lower*)

with As_2O_3 for evidence of apoptosis. Microscopic observation showed that cells treated with As_2O_3 exhibited clear morphological signs of apoptosis. Flow cytometric analysis confirmed that As_2O_3 induced SK-ChA-1 cell apoptosis in a dose-dependent manner. These findings support the results of other previous studies showing a similar effect of As_2O_3 on some human cancer cells [6,9–11,13,20]. Apoptosis can be initiated by a death receptor-mediated pathway or by a mitochondria-mediated pathway [21–24]. In the death receptor-mediated pathway, caspase-8 is activated on the downstream of the death-inducing signaling complex. The active caspase-8 activates downstream executioner caspases (e.g., caspase-3) and/or cleaves Bid into tBid, which leads to the activation of mitochondrial pathway. In the mitochondria-mediated pathway, several apoptogenic molecules, including cytochrome *c* (cyto c) and apoptosis inducing factor (AIF), are released from mitochondria into cytosol as a consequence of mitochondrial membrane permeability transition. Cyto c, once released, forms an apoptosome with Apaf-1 and procaspase-9 in the presence of dATP, resulting in activation of caspase-9 [22,23]. The active subunit of caspase-9

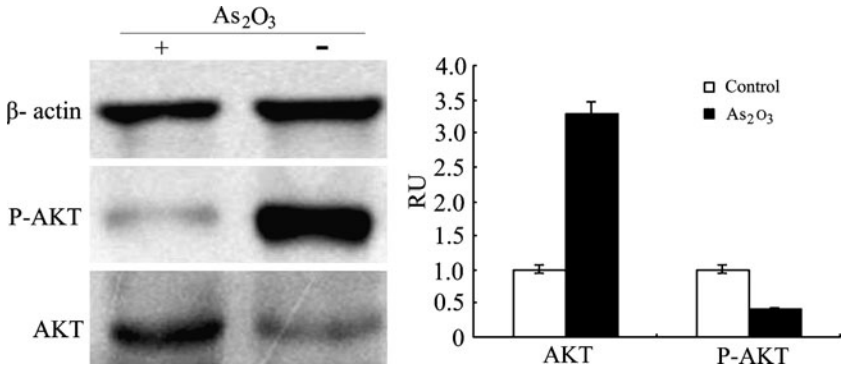

Fig. 5 Effects of As_2O_3 on Akt phosphorylation. Protein expression was examined by Western blot analysis of phospho-Akt and unphosphorylated Akt expression in protein extracts from untreated SK-ChA-1 cells or cells treated with As_2O_3 (2 μmol/L) for 24 h. β-actin was used as loading control. Results are representative of 3 replicate experiments (*left*). The densities of the phospho-Akt and unphosphorylated Akt proteins bands were determined and the relative amount of the target protein was shown as RU (*right*)

further activates downstream pro-caspase-3. Caspase-3 can cleave several cellular proteins, including the PARP protein, initiating a cascade of events that eventually leads to apoptosis [25]. Although the caspase family members are main effectors of apoptosis, they are not required for apoptosis to occur in all systems. AIF is a caspase-independent apoptotic effector which, in the induction of apoptosis, translocates from the mitochondrial intermembrane to the nucleus via the cytosol and causes chromatin condensation and large scale DNA fragmentation, leading to apoptotic cell death [26].

In the caspase inhibitor assay, pretreatment of the cells with z-VAD-fmk partially inhibited or delayed the apoptosis induced by As_2O_3, suggesting that caspase cascade(s) play an important role in As_2O_3-induced apoptosis in SK-ChA-1 cells. Activation of caspase-3, the major effector caspase, and cleavage of PARP, the main substrate of caspases, also support the conclusion. In the present study, we could not observe the activation of caspase-8 in the cells treated with As_2O_3. On the other hand, the activation of caspase-9, decrease of the MMP and the release of cyto c to cytosol were clearly detected. These results suggest that the major pathway is mediated through the release of cytochrome c from mitochondria leading to caspase-9 and -3 activation, but not through the death receptor pathway. In results similar to ours, some author showed the mitochondria-mediated, caspases-dependent apoptosis induced by As_2O_3 occurred in myeloma cells and leukemia cells [27–29]. As pointed out by Platanias [30], this appears to be a common mechanism of As_2O_3-induced cell death in diverse cellular backgrounds. However, we can not exclude the possibility that As_2O_3 trigger apoptosis via other pathways not studied here.

Bcl-2 family proteins, including Bad, Bax, Bid, Bcl-2, and Bcl-xL, are key regulators in mitochondria-mediated apoptosis [31]. Pro-apoptotic Bad and Bax induce apoptotic cell death by promoting mitochondrial release of cytochrome-c, Anti-apoptotic Bcl-2 and Bcl-xL inhibit mitochondria-mediated apoptosis and promote cell survival by preventing cytochrome-c release from the mitochondria [32]. A shift in the balance between anti- and pro-apoptotic bcl-2 family proteins towards the expression of proteins that promote death may be one mechanism underlying apoptotic cell death. Thus, it is possible that As_2O_3 induces mitochondrial release of cytochrome-C by affecting the expression of the bcl-2 family of proteins. Our study demonstrated that the apoptosis that occurs in response to As_2O_3 treatment is accompanied by increased expression of pro-apoptotic genes and reduced expression of anti-apoptotic genes.

Akt plays a key role in tumorigenesis and cancer progression through stimulation of cell proliferation and cell invasion and by inhibition of cell apoptosis [33–35]. It was recently demonstrated that inhibition of Akt phosphorylation decreased cholangiocarcinoma cell viability [36]. This report is in agreement with our observation that As_2O_3 inhibited Akt phosphorylation in cultured cholangiocarcinoma SK-ChA-1 cells. Our results suggest a possible role for Akt inactivation in As_2O_3-mediated cell growth inhibition and induction of apoptosis. However, this is just a preliminary study. More thorough investigations are needed to document the mechanisms by which As_2O_3 inhibits Akt phosphorylation in cholangiocarcinoma cells and its therapeutic implications.

In summary, clinically achievable concentrations of As_2O_3 inhibited cell growth and induced apoptosis in human cholangiocarcinoma cells through a mechanism that may include stimulation of mitochondria-mediated, caspases-dependent apoptotic pathways and Akt inactivation. The study was limited by examination of the antineoplastic effects

and mechanisms of As_2O_3 in only one cell line in vitro. Additional studies are needed to further assess the therapeutic potential of As_2O_3 in cholangiocarcinoma.

References

1. Welzel TM, McGlynn KA, Hsing AW et al (2006) Impact of classification of hilar cholangiocarcinomas (Klatskin tumors) on the incidence of intra- and extrahepatic cholangiocarcinoma in the United States. J Natl Cancer Inst 98:873–875
2. West J, Wood H, Logan RF et al (2006) Trends in the incidence of primary liver and biliary tract cancers in England and Wales 1971–2001. Br J Cancer 94:1751–1758
3. Khan SA, Davidson BR, Goldin R et al (2002) Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. Gut 51(S6):VI 1–9
4. Shaib Y, El-Serag HB (2004) The epidemiology of cholangiocarcinoma. Semin Liver Dis 24:115–125
5. Anderson CD, Pinson CW, Berlin J et al (2004) Diagnosis and treatment of cholangiocarcinoma. Oncologist 9:43–57
6. Zhang TC, Cao EH, Li JF et al (1999) Induction of apoptosis and inhibition of human gastric cancer MGC-803 cell growth by arsenic trioxide. Eur J Cancer 35:1258–1263
7. Chou WC, Dang CV (2005) Acute promyelocytic leukemia: recent advances in therapy and molecular basis of response to arsenic therapies. Curr Opin Hematol 12:1–6
8. Zhu J, Chen Z, Lallemand-Breitenbach V et al (1999) How acute promyelocytic leukaemia revived arsenic. Nat Rev Cancer 2:705–713
9. Shen ZY, Shen WY, Chen MH et al (2003) Reactive oxygen species and antioxidants in apoptosis of esophageal cancer cells induced by As_2O_3. Int J Mol Med 11:479–484
10. Uslu R, Sanli UA, Sezgin C et al (2000) Arsenic trioxide-mediated cytotoxicity and apoptosis in prostate and ovarian carcinoma cell lines. Clin Cancer Res 6:4957–4964
11. Zhang T, Wang SS, Hong L et al (2003) Arsenic trioxide induces apoptosis of rat hepatocellular carcinoma cells in vivo. J Exp Clin Cancer Res 22:61–68
12. Jin HO, Yoon SI, Seo SK et al (2006) Synergistic induction of apoptosis by sulindac and arsenic trioxide in human lung cancer A549 cells via reactive oxygen species-dependent down-regulation of surviving. Biochem Pharmacol 72:1228–1236
13. Shao QS, Ye ZY, Ling ZQ et al (2005) Cell cycle arrest and apoptotic cell death in cultured human gastric carcinoma cells mediated by arsenic trioxide. World J Gastroenterol 11:3451–3456
14. Hoorens A, Van de Casteele M, Kloppel G et al (1996) Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. J Clin Invest 98:1568–1574
15. Kachadourian R, Day BJ (2006) Flavonoid-induced glutathione depletion: potential implications for cancer treatment. Free Radic Biol Med 41:65–76
16. Lecoeur H, Prévost MC, Gougeon ML (2001) Oncosis is associated with exposure of phosphatidylserine residues on the outside layer of the plasma membrane: a reconsideration of the specificity of the annexin V/propidium iodide assay. Cytometry 44:65–72
17. Kroemer G, Galluzzi L, Vandenabeele P et al (2009) Classification of cell death: recommendations of the nomenclature committee on cell death 2009. Cell Death Differ 16:3–11
18. Wang J, Ito T, Ukada N et al (2006) PI3K-AKT pathway mediates growth and survival signals during development of fetal mouse lung. Tissue Cell 37:25–35
19. Shen ZX, Chen GQ, Ni JH et al (1997) Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood 89:3354–3360
20. Gazitt Y, Akay C (2005) Arsenic trioxide: an anti cancer missile with multiple warheads. Hematology 10:205–213
21. Reed JC (2001) Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med 7:314–319
22. Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol 10:369–377
23. van Loo G, Saelens X, van Gurp M et al (2002) The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ 9:1031–1042
24. Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. Science 281:1312–1316
25. Antonsson B (2004) Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem 256–257:141–155
26. Susin SA, Lorenzo HK, Zamzami N et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446
27. McCafferty-Grad J, Bahlis NJ, Krett N et al (2003) Arsenic trioxide uses caspase-dependent and caspase-independent death pathways in myeloma cells. Mol Cancer Ther 2:1155–1164
28. Park MT, Kang YH, Park IC et al (2007) Combination treatment with arsenic trioxide and phytosphingosine enhances apoptotic cell death in arsenic trioxide-resistant cancer cells. Mol Cancer Ther 6:82–92
29. Mahieux R, Pise-Masison C, Gessain A et al (2001) Arsenic trioxide induces apoptosis in human T-cell leukemia virus type 1- and type 2-infected cells by a caspase-3-dependent mechanism involving Bcl-2 cleavage. Blood 98:3762–3769
30. Platanias LC (2009) Biological responses to arsenic compounds. J Biol Chem 284:18583–18587
31. Antonsson B (2004) Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem 256–257:141–155
32. Tsujimoto Y (2002) Bcl-2 family of proteins: life-or-death switch in mitochondria. Biosci Rep 22:47–58
33. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501
34. Testa JR, Bellacosa A (2001) AKT plays a central role in tumorigenesis. Proc Natl Acad Sci USA 98:10983–10985
35. Lawlor MA, Alessi DR (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci 114:2903–2910
36. Wu T, Leng J, Han C et al (2004) The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. Mol Cancer Ther 3:299–307