Combination treatment with arsenic trioxide and sulindac augments their apoptotic potential in lung cancer cells through activation of caspase cascade and mitochondrial dysfunction

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Abstract. Non-steroidal anti-inflammatory drugs (NSAIDs) are known to enhance the responsiveness of tumor cells toward chemotherapeutic drugs and radiation. However, the precise mechanism of synergistic enhancement in tumoricidal activity is not clearly known. Herein, we demonstrate that the combination treatment of arsenic trioxide (As₂O₃) and sulindac resulted in a synergistic augmentation of cytotoxicity toward NCI-H157 lung cancer cells, which was revealed as apoptosis accompanied by chromatin fragmentation and an increase in sub-G₀/G₁ fraction. In addition, combination treatment with As₂O₃ and sulindac increased the catalytic activity of caspase-3, -8, and -9 along with induction of Fas/FasL expression and cytosolic release of cytochrome c. Pharmacologic scavenging study of reactive oxygen species (ROS) revealed that synergistic augmentation of cytotoxicity was achieved by generation of ROS, which might modulate the expression of Bcl-2 family proteins, the activity of caspase-3, and mitochondrial membrane potential transition.

Introduction

Although many new therapeutic maneuvers have been developed in the treatment of cancers, mortality rates of lung cancer patients are currently high and increasing (1). The 5-year survival rate for lung cancer patients with stage Ila disease is 9-25% and with stage IIIb disease is only 3-7%. A number of new agents have become available for the treatment of lung cancer, but until now no chemotherapy regimens have offered a significant advantage over others (2). Therefore, new approaches for the treatment of lung cancer are needed. As a complementary therapy, gefitinib, an inhibitor of epidermal growth factor receptor tyrosine kinase, and Bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor, were clinically tried with chemotherapy regimens to treat lung cancer patients. However, these combination therapies did not have improved efficacy over chemotherapy regimens alone (3,4).

Arsenic trioxide has long been used as an anticancer agent in traditional Chinese medicine (5) and is currently used in the treatment of refractory and relapsed acute promyelocytic leukemia (APL) without severe marrow suppression (6). Some investigators have reported that As₂O₃ induces apoptosis in a variety of human solid tumor cell lines, including carcinomas of the esophagus, stomach, prostate, and ovary as well as neuroblastoma cells (7-10). However, the clinically relevant concentration is a major limiting factor in practical prescription for leukemia patients. The dose range of clinical use is 0.5-2 μM of As₂O₃ in the treatment of leukemia, whereas there should be over 5-10 μM for induction of apoptotic death in many solid tumor cells. As a consequence, the clinical use and efficacy of As₂O₃ have thus far been largely hampered in its universal application to various types of cancer patients, except in patients with APL.

Recently, NSAIDs have attracted much attention after the discovery that sulindac could induce the regression of colon adenomatous polyps in cancer therapy (11). Continuous NSAID administration results in a significant reduction of relative risk for colorectal cancer (12). Sulindac, a structural isomorph of indomethacin, exerts antiproliferative and apoptotic effects which eventually lead to the regression of cancer cells (13,14). NSAIDs are known to enhance the cellular responsiveness of tumors toward chemotherapeutic drugs by apoptosis or anti-angiogenic effect and are also synergistic with radiotherapy by directly increasing cellular radiation sensitivity as well as inhibiting tumor neovascularization (15,16). In this study, we aimed to investigate whether combination treatment with As₂O₃ and sulindac augments their apoptotic potential in NCI-H157 human lung cancer cells.

Materials and methods

Materials. RPMI-1640, fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL Co. (Grand Island,
DEVD-AMC by caspase-3, Ac-IETD-AFC by caspase-8, and Cleavage of fluorogenic substrates, including 100 μM Ac- and used to measure the catalytic activities of caspases (18).

Caspase activity assay
electrophoresis on 1.5% agarose for 3 h at 50 V. DNA was After ethanol precipitations, 5 μg of DNA was subjected to electrophoresis. Genomic DNA from H157 cells was isolated using a Wizard Genomic DNA purification kit (Promega).

Preparation of genomic DNA and electrophoresis

Western blotting

Preparation of cytosolic and mitochondrial fractions
Preparation of cytosolic and mitochondrial fractions was performed according to the previous report (20) with some modifications. In brief, H157 cells were harvested, washed with ice-cold PBS, and then incubated with 50 μM of buffer A (250 mM sucrose, 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin and pepstatin A) on ice for 30 min. Then, cells were disrupted by 20 passages through a 26-gauge needle. The disrupted cells were centrifuged at 750 x g for 10 min. The supernatant was incubated on ice for 30 min and then centrifuged at 10000 x g for 25 min. After centrifugation, the cytosolic fraction was frozen at 70˚C. The pellet containing mitochondria was washed with ice-cold buffer A and then resuspended with cell lysis buffer. The resuspended pellet was incubated on ice for 30 min and then centrifuged at 10000 x g for 25 min. The supernatant was collected as cytosolic fraction of H157 cells.

Statistical analysis
Each experiment was performed at least three times, and all values are represented as means ± SD of triplicates. Student's t-test was used to analyze a statistical significance of the results. Values of p<0.05 were considered as statistically significant.
Results

Combination of As$_2$O$_3$ with sulindac synergistically augmented apoptotic activity of NCI-H157 cells. To test the synergism in cytotoxicity, NCI-H157 cells were treated with As$_2$O$_3$ and sulindac and then viability was measured by MTT assay (Fig. 1A). Neither single exposure of H157 cells to 2.5 μM As$_2$O$_3$ alone nor various concentrations of sulindac alone affected the viability at 48 h. However, combination of 2.5 μM As$_2$O$_3$ with varying doses of sulindac from 2.5 to 20 μM resulted in a significant decrease of the viability of H157 cells in a dose-dependent fashion (*p<0.05). Next, to provide the nature of synergistic cytotoxicity of As$_2$O$_3$ and sulindac, the phenotypic characteristics of apoptosis were examined. Cells were treated with 2.5 μM As$_2$O$_3$ and 5 μM sulindac for 48 h, stained with Hoechst dye 33342 and visualized under fluorescent microscope (Fig. 1B). The nuclei of the control culture as well as of the single-treatment group with As$_2$O$_3$ and sulindac were an oval round shape with homogenous intensity whereas those of cells simultaneously treated with As$_2$O$_3$ and sulindac demonstrated a condensed and fragmented shape with irregularity in staining homogeneity. DNA fragmentation by combination treatment with As$_2$O$_3$ and sulindac was further analyzed as the sub-G$_0$/G$_1$ fraction of cell cycle analysis by flow cytometry in H157 cells stained with PI (Fig. 1C). In contrast to the control culture, combination treatment with As$_2$O$_3$ and sulindac markedly increased the accumulation of the sub-G$_0$/G$_1$ fraction from 5.5% to 23.7%, respectively. These data, collectively, indicate that combined treatment with As$_2$O$_3$ and sulindac may activate apoptotic signaling pathway in H157 cells.

Combination treatment with As$_2$O$_3$ and sulindac increased the catalytic activity of caspase-8. The role of death receptor is well known in apoptosis of lung cancer cells after treatment with various chemotherapeutic agents (21). Thus, we first examined whether combination treatment of As$_2$O$_3$ and sulindac affects the expression of Fas and FasL in H157 cells (Fig. 2A). Cells were treated with 2.5 μM As$_2$O$_3$ and 5 μM sulindac for up to 48 h and lysate was used to carry out Western blotting for Fas and FasL. The date revealed that combination treatment with As$_2$O$_3$ and sulindac resulted in a marked increase in the expression of Fas and FasL proteins in a time-dependent fashion with the same kinetics. We next measured the catalytic...
activity of caspase-8, a downstream target of Fas/FasL, in cells treated with As$_2$O$_3$ and sulindac for 48 h (Fig. 2B). The enzymatic activity of caspase-8 started to increase at 18 h, attained its peak at 36-42 h, and subsequently decreased in cells treated with As$_2$O$_3$ and sulindac. However, single application with either As$_2$O$_3$ or sulindac did not affect the catalytic activity of caspase-8 in cells. Consistent with the enzymatic activity, the expression level of pro-caspase-8 was decreasing at 24 h and was undetectable at 36 h after combination treatment with As$_2$O$_3$ and sulindac. Furthermore, a time-dependent study of Bid expression demonstrated that Bid expression was gradually decreased by combination treatment with As$_2$O$_3$ and sulindac (Fig. 2C). These data indicate that combination treatment with As$_2$O$_3$ and sulindac activates the signaling pathway of extrinsic caspase, including Fas/FasL, caspase-8, and Bid truncation, which may target mitochondrial dysfunction.

**Combination treatment with As$_2$O$_3$ and sulindac increased the catalytic activity of caspase-9.** To confirm the implication of intrinsic caspase cascade, including caspase-9, in apoptotic signaling of H157 cells after combination treatment with As$_2$O$_3$ and sulindac, cells were subjected to the catalytic activity assay for caspase-9 (Fig. 3A). Also, activation of caspase-9 was further confirmed by a gradual decrease in expression level of pro-caspase-9 according to time after combination treatment with As$_2$O$_3$ and sulindac.

We were further interested in the expression of anti- or pro-apoptogenic Bcl-2 family proteins, which is known to affect mitochondrial functions (Fig. 3B) as a downstream modulator of caspase. Combination treatment with As$_2$O$_3$ and sulindac resulted in a gradual increase of pro-apoptogenic Bcl-2 protein whereas it decreased the level of anti-apoptogenic Bcl-X$_L$ in a time-dependent fashion. This result led us to test whether mitochondrial dysfunction occurred in H157 cells treated with 2.5 μM As$_2$O$_3$ and 5 μM sulindac for 48 h. Thus, the cell lysate was fractionated into cytosolic and mitochondrial portions and proteins were separated on 15% SDS-PAGE to immunoblot for cytochrome c. The purity of the mitochondrial fraction was determined with anti-VDAC antibody.
Combination treatment with As2O3 and sulindac increased the catalytic activity of caspase-3. To gain the downstream event of activation of caspases, including caspase-8 and -9, H157 cells were treated with 2.5 μM As2O3 and 5 μM sulindac for up to 48 h, and the proteolytic activity of caspase-3 was determined (Fig. 4A). The enzymatic activation of caspase-3 started after 24 h, attained its peak at 36 h, and was sustained at that level for 48 h in cells treated with As2O3 and sulindac. However, single application with either As2O3 or sulindac did not affect the catalytic activity of caspase-3 of H157 cells. To further confirm the activation of caspase-3, the cleavage of pro-caspase-3 and intracellular biosubstrates, including PARP and ICAD, was measured by Western blot analysis (Fig. 4B). Degradation of pro-caspase-3 by combination treatment with 2.5 μM As2O3 and 5 μM sulindac occurred at 30 h and the immunoreactive band was hardly detectible 42 h after treatment. Cleavage of PARP from 116 to 85 kDa was clearly demonstrated at 30 h after combination treatment with As2O3 and sulindac. Also, ICAD expression started to decrease at 24 h in a time-dependent manner by combination treatment with As2O3 and sulindac.

Antioxidants antagonized the synergistic cytotoxicity of As2O3 and sulindac in H157 cells. To investigate the synergistic mechanism of As2O3 and sulindac, we tested the effect of antioxidants, including NAC and GSH, in H157 cells. Cells were pretreated with either NAC or GAH, followed by the addition of 2.5 μM As2O3 and 5 μM sulindac for 48 h, and viability was measured by MTT assay. As shown in previous data, combination treatment with As2O3 and sulindac resulted in a significant decrease in cell viability (58% of control cells) even though single application of either one did not exert any notable change in cell viability. However, pretreatment with either NAC or GSH significantly inhibited the synergistic cytotoxicity of As2O3 and sulindac in a dose-dependent manner (Fig. 5A). Measurement of intracellular H2O2 was determined fluorimetrically using scopoletin-horseradish peroxidase assay. The data represent the mean ± SD of three independent experiments. *p<0.05; **p<0.01. (B) To verify the direct involvement of reactive oxygen species (ROS), we measured the generation of intracellular H2O2 by scopoletin-horseradish peroxidase assay (Fig. 5B). The data revealed that combination treatment with As2O3 and sulindac induced the
generation of H$_2$O$_2$ (2-fold), which returned to the level of that in control cells by pretreatment with antioxidants, including NAC and GSH.

To further confirm the implication of signaling components of apoptosis, cell lysate was used to perform Western blot analysis. Combination treatment with As$_2$O$_3$ and sulindac induced the expression of apoptogenic Bax whereas it resulted in a decrease in anti-apoptotic Bid and Bcl-X$_L$ (Fig. 5C). Combination treatment with As$_2$O$_3$ and sulindac had no influence on the expression level of Bcl-2 (data not shown). However, the expression levels of Bax, Bid, and Bcl-XL were demonstrated as similar to that of control cells in cells pretreated with NAC and GSH before combination treatment with As$_2$O$_3$ and sulindac. We next measured the effect of antioxidants on the activation of caspase-3. Pretreatment of antioxidants, including NAC and GSH, markedly suppressed the activation of caspase-3, evidenced by the decreased expression of pro-caspase-3 and cleavage of PARP in cells treated with As$_2$O$_3$ and sulindac.

Pretreatment of antioxidants prevented mitochondrial dysfunction by combination treatment with As$_2$O$_3$ and sulindac.

To examine the upstream component of caspase-3 in apoptosis signaling, markers of mitochondrial dysfunction, including mitochondrial membrane potential transition (MPT) and cytosolic release of cytochrome c, were evaluated in cells treated with As$_2$O$_3$ and sulindac. Cells were treated with 2.5 μM As$_2$O$_3$ and 5 μM sulindac for 48 h in the presence or absence of antioxidants, including 10 mM NAC and 15 mM GSH. Then, cells were used to measure the MPT by staining mitochondria with JC-1 under a fluorescence microscope. Mitochondria were mainly located in the cytosol as a punctuated form colored with orange in the control culture (Fig. 6A, a) and cells treated with As$_2$O$_3$ alone (Fig. 6A, b) and sulindac alone (Fig. 6A, c). Simultaneous exposure of H157 cells to As$_2$O$_3$ and sulindac caused a dramatic change in MPT, shown as a diffuse form and mainly located in the cytosol as well as the nuclei (Fig. 6A, d). However, pretreatment of GSH prevented the changes in MPT and morphological features as similarly demonstrated in control cells (Fig. 6A, e).

To provide further evidence of mitochondrial dysfunction, cytosolic release of cytochrome c was examined by Western blotting in both mitochondrial and cytosolic fractions (Fig. 6B). Cytosolic cytochrome c in the cytosolic fraction was markedly increased in cells treated with sulindac only as well as with combination treatment of both. Consistently, the immunoreactive band of cytosolic cytochrome c in cells pretreated with either NAC or GSH remained at a similar level in its intensity to
that of control cells. The purity of mitochondrial fraction was verified by Western blotting with anti-VDAC antibody.

Discussion

NSAIDs are known to enhance tumor cell responses toward various anticancer therapies, including biologic therapy, radiotherapy, and conventional anticancer chemotherapeutic agents. A combination of sulindac and EKI-569, an inhibitor of EGFR tyrosine kinase, remarkably protects intestinal cells from neoplastic transformation (22). NSAIDs also significantly increase the cytotoxicity of anthracyleines and vinca alkaloids in human lung cancer cells and leukemia cell lines (23). The combination of sulindac with paclitaxel and/or cisplatin afforded a synergistic augmentation of cytotoxicity toward lung cancer cell lines (24). In addition, sulindac enhanced cytotoxicity when combined with As2O3 in A549 human lung cancer cells, but the exact mechanism of synergism was not clearly defined (25).

Although As2O3 is known to be effective in the treatment of leukemic cells, especially in APL, there is little evidence of its pharmacologic efficacy in solid tumor cells. Bode and Dong demonstrated that As2O3 induced apoptosis of cancer cells through the consequences of oxidative stress, activation of caspase pathways, and mitochondrial dysfunction (26). Arsenic trioxide directly condenses mitochondrial matrix and decreases mitochondrial transmembrane potential (MMP) to trigger apoptosis via the release of cytochrome c from mitochondria and the subsequent activation of the caspase proteins (27,28). Also, treatment of As2O3 results in reduction of GSH and thereby increases the intracellular ROS level in certain APL cell lines (29).

In this study, we showed that combination treatment with As2O3 and sulindac enhanced the activity of intrinsic and extrinsic caspase cascades through ROS generation. Furthermore, combination treatment also resulted in loss of MMP, changes in expression of Bcl-2 family proteins and cytosolic release of cytochrome c. Mitochondria are known as an important regulator of apoptosis (30). It undergoes a series of consequence changes during apoptotic death of cells. A loss of MMP together with the permeability transition pore induces the cytosolic release of cytochrome c, which further activates caspase-3, following activation of caspase-9. In accordance with previous reports of apoptosis signaling, our data demonstrated that combination treatment resulted in perturbation of mitochondrial functions in H157 cells.

Recent evidence suggests that sulindac engages the mitochondrial pathway involving caspase-9 and Bax in induction of apoptotic effects. Similarly, As2O3 can induce apoptosis via upregulation of intracellular ROS, such as H2O2, which cause depolarization of the mitochondrial transmembrane and subsequent caspase-3 activation. These pathways may account for the interactions between As2O3 and sulindac in our experimental model. The molecular mechanism of synergism in the combination of As2O3 and sulindac was involved in the generation of ROS because the apoptotic death of H157 cells by combination treatment was inhibited by scavenging H2O2 with NAC and GSH, consequently restoring the MPT, which preceded the caspase-3 activation, DNA fragmentation, and morphologic features of apoptosis.

Consistent with the report of Finucane et al. (31), we also speculate that the induction of apoptotic Bcl-2 family protein, such as Bax, together with decreased levels of anti-apoptotic Bcl-2 family protein, such as Bid and Bcl-XL, may contribute the release of cytochrome c and generation of ROS in H157 cells treated with As2O3 and sulindac. Single treatment with sulindac could induce the cytosolic release of cytochrome c even though the MPT was not changed at all. This result may indicate the possibility that MPT is not always required for cytosolic release of cytochrome c or that cytosolic release of cytochrome c is not only a limiting condition for caspase-3 activation, followed by formation of apoptosome complex.

Up to 10-fold higher concentrations of As2O3 are required to induce apoptosis in non-APL tumor cells, which is generally unacceptable in the clinic because of higher toxicity. As a consequence, clinical use of As2O3 has thus far been largely limited to patients with APL. Our data suggest that combination treatment with As2O3 and sulindac may be useful in practical application in the treatment of solid tumors, including lung cancer, as an alternate anticancer strategy for circumventing the serious general toxicity of anticancer chemotherapeutic agents, such as As2O3.

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References


