Inhibition of Mitochondrial Respiration

A NOVEL STRATEGY TO ENHANCE DRUG-INDUCED APOPTOSIS IN HUMAN LEUKEMIA CELLS BY A REACTIVE OXYGEN SPECIES-MEDIATED MECHANISM*

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Cancer cells are under intrinsic increased oxidative stress and vulnerable to free radical-induced apoptosis. Here, we report a strategy to hinder mitochondrial electron transport and increase superoxide (O2•−) radical generation in human leukemia cells as a novel mechanism to enhance apoptosis induced by anticancer agents. This strategy was first tested in a proof-of-principle study using rotenone, a specific inhibitor of mitochondrial electron transport complex I. Partial inhibition of mitochondrial respiration enhances leakage from the transport chain, leading to an increase in O2•− generation and sensitization of the leukemia cells to anticancer agents whose action involve free radical generation. Using leukemia cells with genetic alterations in mitochondrial DNA and biochemical approaches, we further demonstrated that As2O3, a clinically active anti-leukemia agent, inhibits mitochondrial respiratory function, increases free radical generation, and enhances the activity of another O2•−-generating agent against cultured leukemia cells and primary leukemia cells isolated from patients. Our study shows that interfering mitochondrial respiration is a novel mechanism by which As2O3 increases generation of free radicals. This novel mechanism of action provides a biochemical basis for developing new drug combination strategies using As2O3 to enhance the activity of anticancer agents by promoting generation of free radicals.

Mitochondria are essential cellular organelles that play central roles in energy metabolism and apoptosis. During oxidative phosphorylation, electrons are delivered through the mitochondrial respiratory complexes, and a proton gradient is established across the inner mitochondrial membrane as the energy source for ATP production. One important biochemical event associated with this metabolic process is the production of superoxide radical (O2•−). When an electron escapes from the mitochondrial electron transport chain, especially at complex I or III, it may react with a molecular oxygen to form O2•− (1, 2). Because O2•− is constantly generated during cellular metabolism and can be converted to hydrogen peroxide (H2O2) and other reactive oxygen species (ROS),1 mitochondria are considered the major source of cellular ROS (3, 4). Because ROS are chemically reactive and potentially toxic to the cells when accumulated to high levels, these chemical species need to be promptly eliminated from the cells by a highly regulated antioxidant system, which include metabolic enzymes such as superoxide dismutase (SOD), various peroxidases, and other redox molecules.

Under physiological conditions, the maintenance of an appropriate level of intracellular ROS is important in keeping redox balance and signaling cellular proliferation (5–9). However, an overproduction of ROS or a suppression of cellular ability to eliminate ROS from the cells would result in a significant increase of intracellular ROS, leading to cellular damage, including lipid peroxidation, oxidative DNA modifications, protein oxidation, and enzyme inactivation. These damages can ultimately cause cell death if the ROS stress is severe and persistent. Although the cell-damaging effect of free radicals has been associated with various pathological processes, this may also provide a possibility to kill cancer cells if one could preferentially induce ROS accumulation in the malignant cells with appropriate agents. Because cancer cells are in general metabolically active, produce high levels of ROS, and are under intrinsic oxidative stress, the malignant cells are more vulnerable to further oxidative stress by exogenous ROS-generating agents (10–15). It has been postulated that when ROS stress reaches a certain threshold, an apoptotic program is triggered to kill the cancer cells. This hypothesis is supported by the observations that the anticancer agent 2-methoxyestradiol (2-ME) inhibits the O2•−-eliminating enzyme SOD and causes an accumulation of superoxide radical in cancer cells, leading to a preferential killing of the malignant cells and an enhancement of photodynamic therapy (16–18).

In addition to the essential role in ATP synthesis and ROS metabolism, mitochondria also play a critical role in apoptosis. Upon receipt of apoptotic stimuli, either externally or internally generated, respective signaling pathways are initiated in the cells and often converged upon the mitochondria to promote release of cytochrome c and other apoptotic factors to the cytoplasm. This is associated with changes in the mitochondrial ultrastructure, membrane permeability, and transmembrane potential (19). A direct damage to the mitochondrial membrane by ROS also causes a leak of cytochrome c (16, 20–22). Cytochrome c is normally localized in the mitochondrial intermembrane space and is part of the mitochondrial electron transport chain essential for aerobic ATP generation. However, once cytochrome c is released, it can trigger the caspase-dependent apoptotic cascade, which results in the breakdown of cells.

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1 The abbreviations used are: ROS, reactive oxygen species; O2•−, superoxide radical; 2-ME, 2-methoxyestradiol; PLL, chronic lymphocytic leukemia; Rot, rotenone. ND1, NADH dehydrogenase 1; SOD, superoxide dismutase; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; PI, propidium iodide; NAC, N-acetylcysteine.
tochrome c is released into the cytosol, it binds apaF-1 in the presence of dATP or ATP and activates procaspase-9, which in turn activates the downstream caspases and triggers the apoptotic cascade (23, 24).

It has long been recognized that cancer cells may have certain alterations in the mitochondria, leading to increased glycolysis, even in the presence of normal oxygen tension (25). This “respiration injury” would predict a low coupling efficiency of the mitochondrial electron transport, and an increased likelihood of electron leakage during respiration in the cancer cells, leading to O$_2^-$ radical formation and increased oxidative stress. These biochemical characteristics suggest a possibility that the metabolism-based targeting of cancer cells may be amenable to therapeutic intervention and may serve as a potential target for cancer therapy. The objective of this study was to test a new approach to enhance drug-induced apoptosis by a drug combination strategy that increases mitochondrial generation of O$_2^-$ radical and at the same time inhibits its elimination.

Because the overall cellular O$_2^-$ content is determined by the balance between O$_2^-$ generation and elimination, interference of both processes is expected to cause a severe accumulation of O$_2^-$ radical and likely to have a synergistic effect against cancer cells. In the present study, we used rotenone, a known inhibitor of the mitochondrial respiratory complex I, in combination with the SOD inhibitor 2-ME to test their effect on cellular superoxide levels and possible synergistic activity against cancer cells in vitro. The potential therapeutic implications of this approach were further investigated using clinically relevant anticancer agents whose action involves ROS generation.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Cultures**—Human monoblastic ML-1 and lymphoblastic T-cell Jurkat lines were cultured at 37 °C in a humidified atmosphere with 5% CO$_2$ in RPMI 1640 medium supplemented with 10% fetal bovine serum. Primary leukemia cells were isolated from patients diagnosed as having B-cell CLL according to the National Cancer Institute (NCI, National Institutes of Health) criteria for the diagnosis of CLL (26). CLL cells were isolated from peripheral blood by density gradient centrifugation as previously described (27). Briefly, fresh blood samples were diluted with 3 volumes of cold (4 °C) phosphate-buffered saline (PBS) and then layered over 10 ml of Ficoll-Hypaque mixture (specific gravity, 1.077 g/ml). After centrifugation at 400 × g for 20 min, theuffy coat layer containing leukemia cells was recovered, washed twice with sterile PBS, and resuspended in RPMI 1640 culture medium supplemented with 10% fetal bovine serum. Drug incubations were started after the cells were pre-cultured in fresh medium for 24 h. Incubation of CLL cells in fresh medium (no drug) for 48 h resulted in about 5–30% of the cells being annexin-V positive, indicating various degrees of “spontaneous” apoptosis among CLL samples from different patients. This is consistent with the observations by Robertson et al. (28). In all experiments, the drug-induced apoptosis was calculated by subtracting “spontaneous” apoptosis from total apoptosis of the respective samples.

**Reagents**—Hydroethidine and Mitotracker Green were obtained from Molecular Probes (Eugene, OR). Human PARP antibody and cytochrome c antibody were obtained from BD Pharmingen (San Diego, CA). Antibody to β-actin and all other reagents, unless otherwise stated, were obtained from Sigma Chemical Co.

**Western Blot Analysis**—Cytosolic protein extracts were prepared as previously described (29). Briefly, cells were collected by centrifugation at 300 × g for 5 min at 4 °C and washed with ice-cold PBS. The cell pellets were then resuspended in 500 μl of lysis buffer (20 mM Hepes-KOH, pH 7.5, 250 mM sucrose, 70 mM mannitol; 1.5 mM MgCl$_2$, 10 mM KCl, 10 μl/ml leupeptin, and 10 μl/ml digitonin). After a 10-min incubation at 25 °C, the samples were spun at 14,000 × g for 15 min, and the supernatants containing cytosolic proteins were stored at −80 °C until analysis by polyacrylamide gel electrophoresis (SDS-PAGE). The protein extracts were subjected to standard SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and probed for various proteins (cytochrome c, PARP, and β-actin) using appropriate antibodies as described individually in the figure legends. The bound primary antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal enhanced chemiluminescence kit (ECL, Pierce, Rockford, IL).

**Analysis of Cellular Superoxide and Mitochondrial Transmembrane Potential Δψm**—Cellular superoxide was measured by a flow cytometry analysis using dihydroethidium as the fluorescent dye relatively specific for superoxide (30). Dihydroethidium was dissolved in Me$_2$SO (100 mg/ml stock) and further diluted with PBS at 1:10,000. The diluted dye was added into the cell culture at a final concentration of 50 nM and incubated at 37 °C during the last 60 min of the drug treatment. The cells were washed twice in PBS and then analyzed with a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA) as described previously (16). Rhodamine-123, a cationic voltage-sensitive probe that reversibly accumulates in mitochondria (31), was used in the present study to detect changes in transmembrane potential (Δψm). Exponentially growing cells were incubated with rotenone, 2-ME, or both in combination as indicated in the figure legends. Cells were labeled with 1 μM rhodamine-123 at 37 °C for 60 min before the end of the drug incubation. After washing, the samples were analyzed by flow cytometry as described previously (16).

**Assessment of Apoptosis by Annexin-V Staining and Analysis of Cellular DNA Contents**—Cells were stained with annexin-V-FITC for exposure of phosphatidylserine on the cell surface as an indicator of apoptosis, following the manufacturer’s instruction (BD Biosciences). Briefly, after cells were treated with the indicated compounds, ~1 × 10$^6$ cells were collected, washed with a binding buffer (10 mM Hepes-KOH, pH 7.4, 140 mM NaCl, 1.8 mM CaCl$_2$), and then resuspended in 10 μl of the binding buffer containing 10 μl of annexin-V-FITC. After 15 min of incubation at room temperature in the dark, the cells were washed with the binding buffer (without annexin-V), resuspended in 300 μl of buffer containing propidium iodide (PI), and then analyzed by a flow cytometer. Data acquisition and analysis were performed using a BD Biosciences FACSCalibur flow cytometer with CellQuest software.

Cells that were positively stained by annexin-V-FITC only (early apoptosis) and positive for both annexin-V-FITC and propidium iodide (late apoptosis) were quantitated and both subpopulations were considered as overall apoptotic cells. To analyze cellular DNA contents, cells were collected after drug incubation as indicated in the figure legends, fixed in 70% ice-cold ethanol, and kept at −20 °C for at least 14 h. The fixed cells were washed twice with PBS, stained with propidium iodide (50 μg/ml) in PBS containing 0.2% Tween 20 and 2.5 μg/ml RNase at 4 °C overnight, and analyzed by flow cytometry. The profiles of cellular DNA contents indicate the distribution of the cells in different phases of the cell cycle and any possible DNA loss due to DNA fragmentation during apoptosis. The cells with a DNA content less than a G1 cellular DNA content were considered as sub-G$_0$ population and considered as apoptotic cells (32).

**Measurement of Mitochondrial Respiration Activity in Cultured Cells**—Oxygen consumption by intact cells was measured as an indication of the mitochondrial respiration activity. After appropriate drug treatment, the cells were resuspended in 1 ml of fresh culture medium pre-equilibrated with 21% oxygen and then placed in the sealed respiration chamber equipped with a thermostat control and a micro-stirring device (Oxytherm, Hansatech Instrument, England). Oxygen consumption was measured polarographically at 37 °C with the Clark-type oxygen electrode disc, using the conditions recommended by the manufacturer. The oxygen content in the suspension medium was constantly monitored, and the signals were integrated using the software supplied by the manufacturer. The oxygen contents in the starting medium were normalized assuming an O$_2$ concentration of 220 μM in air-saturated medium at 37 °C (33). Respiration rate is expressed as nanomoles of O$_2$ consumed as a function of time (min).

**DNA Sequence Analysis of Mitochondrial ND1 Gene**—DNA was isolated from the HL-60 human leukemia cell line and its respiratory-deficient subclone C6F–/– cells using a method previously described (34). Briefly, cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml Proteinase K, and then incubated at 45 °C in a water bath for 16 h. Following 7000 × g centrifugation, the cell lysates were washed with 1 ml of 95% ethanol containing 0.1% chloroform/isooamyl alcohol (25:24:1, Promega, Madison, WI) and rotated at ambient temperature for 10 min. Samples were centrifuged at 2000 × g for 15 min. The aqueous phase was transferred to a fresh tube, added 150 mM NaAc and 10 mM MgCl$_2$, and mixed with 3 volumes of ice-cold ethanol. After precipitated overnight at −20 °C, the DNA was recovered by centrifugation at 10,000 × g for 30 min at −8 °C. The DNA was dissolved twice with 100% ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). To analyze the DNA sequence of the mitochondrial ND1 gene, a region of the ND1 gene was amplified under standard PCR conditions using the specific primers as previously described (35).

**Enhance Apoptosis by ROS-mediated Mechanism**

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Briefly, amplifications were performed in 50-μl reactions comprising ~50 ng of DNA, 0.1 mM dNTPs, 1× PCR Gold Buffer, 1.5 mM MgCl2, 0.5 μM each primer, 2.5 units of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and nuclease-free H2O (Promega, Madison, WI). Primer sequences used for amplification of a region of the ND1 gene (amino acids 3304–3836) were as follows: sense, 5'-AACATA-CCCATGCCAACCCT3'; antisense, 5'-GGCAGGAAGTAACAGAGGT-G3' (Sigma-Genosys, The Woodlands, TX). PCR reactions were amplified in a Robocycler (Stratagene, La Jolla, CA) using the following cycling protocol: 94°C for 3 min, 55°C for 3 min, 72°C for 1 min (first cycle); 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles. The final cycle was prolonged to allow a 7-min extension at 72°C. 10% of each PCR product was electrophoresed along with 500 ng of a 100-bp ladder (Invitrogen, Carlsbad, CA) on a 1.2% agarose gel at 100 V for 45 min to confirm product purity and correct size. The remaining portion of the PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified products were sequenced using the same primers used for PCR amplification (SeqWright, Inc., Houston, TX). Sequencing results were aligned with the revised Cambridge reference sequence, GenBankTM accession NC-001807, using the pairwise BLAST alignment method (National Center for Biotechnology Information, available at www.ncbi.nlm.nih.gov). Amino acid changes were determined using the MitoAnalyzer Tool (National Institute of Standards and Technology, available at www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html).

DNA fragmentation—DNA fragmentation assay was performed according to the method described previously (16). HL-60 cells were treated with the indicated compounds in the presence or absence of an antioxidant (N-acetylcyesteine). Cells (1×106) were washed once with PBS (4°C, pH 7.4) and collected by centrifugation at 250 × g for 5 min. The cell pellets were then digested in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% sodium deoxycholate, and 0.1 mg/ml Proteinase K overnight. For analysis, 30 μg of DNA was loaded on a 1.8% agarose gel containing 10 μg/ml ethidium bromide. Electrophoresis was performed in 0.5× Tris borate-EDTA buffer (18 mM Tris-HCl (pH 8.0), 18 mM boric acid, and 1 mM EDTA) at 70 V for 3 h. After treatment with RNase A (final concentration, 100 μg/ml) overnight, DNA bands were visualized under ultraviolet light and photographed.

RESULTS

Recent studies suggest that cancer cells are more active in free radical production and are under increased oxidative stress compared with their normal counterparts (15). We demonstrated that this holow better than primary leukemia cells isolated from patients with chronic lymphocytic leukemia (CLL) were compared with normal lymphocytes from healthy donors. As shown in Fig. 1A, quantitation of cellular O2 radical by flow cytometry analysis revealed that the average O2 radical content in CLL cells was 18.9 ± 4.8 arbitrary units (fluorescence intensity, n = 12 patients), significantly higher than that of the normal lymphocytes (6.4 ± 1.3, p < 0.001). The intrinsic oxidative stress makes CLL cells much more sensitive than normal lymphocytes to 2-ME, which blocks O2 elimination. Further blockage of respiration was observed by the cells. Further blockage of electron flow, leading to a one-electron transfer from the electron transport chain to molecular oxygen and the formation of superoxide (2). We used rotenone, a known inhibitor of mitochondrial electron transport complex I (36, 37), to test its ability to increase superoxide generation and to enhance the activity of 2-ME. The rationale for this experimental design is shown in Fig. 2A. We postulated that a partial blockage of complex I function by a low concentration of rotenone might divert a portion of the electron flow to molecular oxygen and thus increase the production of O2. Addition of 2-ME to the rotenone-treated cells would inhibit the elimination of O2 by SOD and caused a further increase of O2. This indeed occurred when human leukemia cells were tested. As shown in Fig. 2B, incubation of Jurkat cells with 50 nM rotenone for 2 h caused a partial inhibition of mitochondrial electron transport, as evidenced by a significant decrease in oxygen consumption by the cells. Further blockage of respiration was seen with higher concentrations of rotenone (Fig. 2C). As expected, relatively low concentrations of rotenone (50–250 nM) caused an increase in of O2 generation in Jurkat cells (Fig. 2D) and ML-1 cells (Fig. 2E).

A sub-toxic concentration of 2-ME (0.3 μM) was then used to test its ability to further increase O2 radical accumulation in the rotenone-treated cells and to evaluate the combined cytotoxic activity of both compounds. As demonstrated in Fig. 3 (A and B), 0.3 μM 2-ME alone caused only a moderate increase of cellular O2 radical. Consistent with this observation, 0.3 μM 2-ME showed a minimum apoptotic effect in these leukemia cells, as evidenced by a small amount of DNA fragmentation.
Combination of the two sub-toxic concentrations of rotenone and 2-ME resulted in an additive accumulation of O$_2^-$ radical in both Jurkat and ML-1 cells (Fig. 3, A and B). This increase of superoxide was associated with a substantial increase of apoptosis, as demonstrated by a loss of mitochondrial transmembrane potential (Fig. 3C), release of cytochrome c and cleavage of poly(ADP)-ribosyltransferase (Fig. 3D), and appearance of cells with a sub-G$_1$ DNA content (Fig. 3E) after a 6-h incubation with 50 or 250 nM, respectively.

(data not shown). Combination of the two sub-toxic concentrations of rotenone and 2-ME resulted in an additive accumulation of O$_2^-$ radical in both Jurkat and ML-1 cells (Fig. 3, A and B). This increase of superoxide was associated with a substantial increase of apoptosis, as demonstrated by a loss of mitochondrial transmembrane potential (Fig. 3C), release of cytochrome c and cleavage of poly(ADP)-ribosyltransferase (Fig. 3D), and appearance of cells with a sub-G$_1$ DNA content (Fig. 3E). Quantitation of apoptotic cells by annexin-V/PI double staining using flow cytometry analysis further confirmed the combination of 2-ME and rotenone had more than additive activity against leukemia cells (Table I). In Jurkat cells, incubation with 50 nM rotenone or 0.3 μM 2-ME alone for 24 h caused apoptosis in 5 and 16% of the cells, respectively. Combination of both compounds resulted in 37% apoptotic cells. This greater than additive cytotoxic activity was also observed in ML-1 cells (Table I). It is of interest to note that rotenone and 2-ME caused an additive accumulation of O$_2^-$ in the cells, whereas their apoptotic effect was more than additive. Thus, the extent of apoptosis may not be proportional to the degree of free radical accumulation. It is possible that in addition to ROS accumulation, other mechanisms could also be involved in the killing of leukemia cells.

The more than additive activity of 2-ME and rotenone combination was also demonstrated by a flow cytometry assay of DNA contents. As shown in Fig. 3E, only a small number of cells with sub-G$_1$ DNA content, an indication of loss of fragmented DNA during apoptosis, was observed in ML-1 cells.
treated with rotenone or 2-ME alone at 24 h. In contrast, combination of both compounds induced apoptosis in 30% of the cells by 24 h, and by 48 h the majority of the cells became apoptotic. The loss of intact cells from all phases of the cell cycle suggests that the drug-induced apoptosis is independent of cell cycle, consistent with a free radical-mediated mechanism, which damages the mitochondrial membrane integrity regardless of the status in the cell cycle. Similar results were obtained in experiments with Jurkat cells (data not shown). We then tested if rotenone could enhance the activity of 2-ME against primary leukemia cells isolated from CLL patients. Fig. 3F summarizes the results of such experiments in CLL cells from eight patients, using annexin-V/PI double staining assay as an indicator of apoptosis. 2-ME, rotenone, and their combination induced apoptosis in 16, 15, and 44% of the cells, respectively. Again, a more than additive effect was observed when both compounds were combined.

Because the increase of superoxide generation by rotenone was able to enhance 2-ME-induced apoptosis, we further tested if rotenone could enhance the cytotoxic activity of other agents that involve in free radical generation. Doxorubicin and ionizing radiation (IR), two modalities commonly used in the clinical treatment of cancer, were used for this evaluation. As demonstrated in Fig. 4A, rotenone was able to enhance the ability of doxorubicin and IR to induce apoptosis. In the case of rotenone and doxorubicin, the observed combined activity was more than the calculated additive effect. Rotenone and doxorubicin alone caused 11 and 27% apoptotic cells, respectively. Although the expected additive effect was calculated to be 35% (survival percentage = 0.89 × 0.73 = 0.65; apoptosis percentage = 1 − 0.65 = 0.35), the percentage of apoptotic cells actually observed in this sample was 53%. In the case of rotenone and IR, the combined effect appeared to be additive. In a separate experiment, incubation of Jurkat cells with cisplatin (2 μM, 38 h) caused 24% apoptotic cells (annexin-V assay). Addition of 50 nM of rotenone during the last 14 h of cisplatin incubation increased the apoptotic response to 50% (Fig. 4B). This concentration of rotenone alone did not cause a detectable apoptosis under the same incubation conditions. Thus, the apoptosis-promoting effect of rotenone is not limited to 2-ME. It is possible to use rotenone to increase ROS generation and enhance the activity of a variety of anticancer agents, whose mechanism of action involves ROS generation.

Because rotenone is a toxic compound and has not been used in clinic as a cancer therapeutic agent, we further explored the possibility of using arsenic trioxide (As$_2$O$_3$), an ROS-generating anticancer agent (38–40) currently used in clinical treatment of cancer. The mechanism by which As$_2$O$_3$ increases ROS remains unclear. Because the mitochondrial respiratory chain is the major site of ROS production under physiological conditions, we first tested if As$_2$O$_3$ might alter mitochondrial respiration to promote free radical generation. As$_2$O$_3$ was found capable of inhibiting mitochondrial respiration, as evidenced by a substantial decrease of oxygen consumption in the As$_2$O$_3$-treated cells (Fig. 5A). The inhibition of respiration by arsenic trioxide seems to be a primary event, because this occurred as early as 3 h after drug addition (1 μg/ml), whereas cell death was detected after 24 h. Additional experiments suggest that this inhibition seems to occur upstream of complex IV in the mitochondrial respiratory chain, because As$_2$O$_3$ did not affect the respiration rate of complex IV when its specific substrates (N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride and ascorbate) were used. Furthermore, in the presence of the complex I inhibitor rotenone, As$_2$O$_3$ caused a further inhibition (data not shown), suggesting that As$_2$O$_3$ and rotenone may act in series. Similar to rotenone, arsenic trioxide also caused an increase of superoxide radical generation in a time-dependent manner (Fig. 5B). These data suggest that it might be possible to use As$_2$O$_3$ instead of rotenone for combination with other anticancer agents to enhance anticancer activity. Indeed, when 2-ME was used in combination with arsenic trioxide, a more than additive cytotoxic activity was observed in Jurkat cells (Fig. 5C). An incubation with 2-ME (1 μM) or As$_2$O$_3$ (1 μg/ml) alone caused apoptosis in 36 and 8% of the cells at 24 h, respectively, whereas their combination resulted in 63% apoptotic cells.

Table I

Enhancement of apoptotic response by rotenone in human leukemia cells treated with 2-ME

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Jurkat</th>
<th>ML-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.1 ± 0.2</td>
<td>4.6 ± 1.2</td>
</tr>
<tr>
<td>Rotenone</td>
<td>5.2 ± 1.0</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>2-ME</td>
<td>16.3 ± 3.2</td>
<td>14.8 ± 3.5</td>
</tr>
<tr>
<td>2-ME + rotenone</td>
<td>37.5 ± 2.1</td>
<td>32.3 ± 8.6</td>
</tr>
</tbody>
</table>

Fig. 4. Sensitization of the cells to anticancer agents by rotenone. A, Jurkat cells were treated with 2 μM doxorubicin (Dox, 24 h), 100 nM rotenone (Rot, 24 h), 5 Gy ionizing radiation (IR), or the indicated combinations. In the case of rotenone/IR combination, rotenone was added 8 h after cells were exposed to IR. B, Jurkat cells were treated with 2 μM cisplatin (CDDP, 38 h) in the presence or absence of 100 nM rotenone (added 14 h after cisplatin exposure). Apoptosis was measured by flow cytometry analysis using annexin-V/PI double staining and expressed as the percentage of annexin V-positive cells (mean ± S.D.) from five separate experiments.
As$_2$O$_3$ cytotoxic action. The respiration-deficient cell line (C6F/H9267/H11002) was isolated from ethidium-treated HL-60 cells and subcloned by the limited dilution method as described previously (41). DNA sequencing analysis revealed that the mitochondrial DNA (mtDNA) of C6F/H9267/H11002 cells contained a G insertion at nucleotide position 3401 (Fig. 6A), which causes a coding frame-shift, starting from the 32nd amino acid of the NADH dehydrogenase 1 (ND1). C6F/H9267/H11002 cells also contained multiple nucleotide substitutions in other coding regions of the mtDNA. These mitochondrial DNA alterations caused a complete loss of respiration in C6F/H9267/H11002 cells and inability to consume oxygen (Fig. 6B), although the cells still contain mitochondrial mass comparable to the parental cells (measured by Mitotracker Green, Molecular Probe). Due to the lack of mitochondrial respiration, the C6F/H9267/H11002 cells depend on glycolysis as the energy source and, as expected, produced substantially less superoxide radicals (20% of the control cells). When C6F/H9267/H11002 cells were incubated with As$_2$O$_3$, they were relatively resistant to the drug (Fig. 6C), suggesting that the mitochondrial respiratory function is important for the action of As$_2$O$_3$. The SOD inhib-
Enhance Apoptosis by ROS-mediated Mechanism

**DISCUSSION**

Due to the essential roles of mitochondria in energy metabolism and apoptosis, there are growing interests in the recent years to explore new approaches to interfering/modulating mitochondrial function as a mean to kill cancer cells. Certain structural and biochemical alterations of mitochondria in cancer cells make it possible to develop strategies to preferentially affect cancer cells. For instance, mitochondrial hyperpolarization, a shared feature of many cancer cells, allows a preferential accumulation of drug molecules with a delocalized positive charge such as F16 in the mitochondria of the tumor cells, leading to a selective inhibition of tumor cell growth (43). Another common feature of cancer cells is their intrinsic oxidative stress (15, 42), presumably due to changes in mitochondrial energy metabolism of free radical production. These biochemical characteristics makes the cancer cells more vulnerable to damage by additional ROS stress, either through inhibiting superoxide elimination or by adding exogenous ROS (16, 44).

The cell-damaging property of free radicals and the increased free radical generation in cancer cells provide a possibility to design new strategies to preferentially kill cancer cells through the ROS-mediated mechanism. The rates of endogenous free radical generation and elimination are two important factors that determine the overall cellular ROS levels. Compounds that affect either one of these two processes can impose oxidative stress to cells. A combination of two agents to simultaneously increase endogenous ROS generation and inhibit ROS elimination would be a logical strategy to cause severe ROS accumulation in cancer cells. Because bifurcation of electrons from the mitochondrial complexes to molecular oxygen is a primary source of endogenous ROS (1, 2), agents that interfere with the electron transport process can be used to divert electron flow and increase ROS generation. In the present study, the known inhibitor of complex I, rotenone, was first used in the initial proof-of-principle experiments. Indeed, rotenone caused a partial diversion of electron flow to oxygen and enhanced the metabolic production of radicals. This is in agreement with a recent report that rotenone induces apoptosis via enhancing the amount of mitochondrial ROS (45). In the present study, we used a sub-toxic concentration of rotenone to cause increased O$_2^-$ generation and 2-ME to inhibit SOD and radicals appears to be a critical elimination; this effectively enhanced cellular O$_2^-$ from four separate experiments are shown in Fig. 7A. Impor-
tantly, combination of As$_2$O$_3$ and 2-ME no longer showed any enhanced activity in C6F/p$^-$ cells (Fig. 6C), further confirming that the mitochondrial generation of superoxide radical by the respira-
tion chain is essential for the anti-leukemia activity by the drug combination. No significant increase of ROS was detected when C6F/p$^-$ cells were incubated with 2-ME and As$_2$O$_3$ (data not shown).

To further test if the increase of free radical was essential for the enhanced anti-leukemia activity when 2-ME and As$_2$O$_3$ were combined, we again used the antioxidant NAC to evaluate its effect on the drug-induced apoptosis. As shown in Fig. 7A, addition of 10 mM NAC to the drug-treated (1 $\mu$m 2-ME plus 1 $\mu$g/ml As$_2$O$_3$) cells effectively reverse the cytotoxic effect of the drug combination, as indicated by the disappearance of the annexin-V-positive cells. The protective effect of NAC was further evidenced by a DNA fragmentation assay (Fig. 7B). The quantitative data on apoptosis by annexin-V/PI analysis from four separate experiments in HL-60 cells are shown in Fig. 7B. It is clear that the antioxidant NAC protected the cells from the cytotoxic effect of 2-ME and As$_2$O$_3$, suggesting that ROS is an important mediator of the drug action under the drug combination conditions.

**FIG. 7. Effect of antioxidant N-acetylcysteine on apoptosis induced by As$_2$O$_3$ and 2-ME.** A, HL-60 cells were incubated with 2-ME (1 $\mu$m), As$_2$O$_3$ (1 $\mu$g/ml), or their combination in the presence or absence of N-acetylcysteine (NAC) as indicated. NAC (10 mM) was added to the cell culture 60 min before addition of 2-ME and As$_2$O$_3$. After 24 h of drug incubation, cells were collected, and apoptosis was assayed by flow cytometry analysis using annexin-V and PI staining. Quantitative data on apoptotic cells (sum of lower right quadrant and upper right quadrant) from four separate experiments are shown in B as means $\pm$ S.E. C, HL-60 cells were treated with the indicated compounds as in A, and cellular DNA was isolated and analyzed on a 1.8% agarose gel. DNA fragmentation was visualized by UV light after the gel was stained with ethidium bromide. Results are representative of three separate experiments. The number below each lane indicates the relative amount (light density) of the intact DNA remained near each sample well.
malfuction of the mitochondria are likely factors contributing to the increased production of ROS. It has been known for a long time that cancer cells have increased glycolytic activity (Warburg effect), perhaps reflecting a less efficient ATP generation in the mitochondria due to respiration injury, and thus forcing the cancer cells to increase glycolysis to produce the ATP required for cellular functions (25, 47). A lower mitochondrial respiratory coupling efficiency may lead to more leakage of electrons from the transport complexes to react with oxygen, forming superoxide radicals. The increased oxidative stress in cancer cells is likely to make them more vulnerable to agents that generate ROS or interfere with mitochondrial function.

Arsenic trioxide is currently used in the clinical treatment of cancer, especially for the treatment of patients with refractory acute promyelocytic leukemia (48–51). This compound has been shown to cause increase ROS in cells, although the precise mechanism was not clear (38–40). We recently demonstrated that the degree of 2-ME-induced apoptosis is significantly correlated with the extent of the drug-induced increase in O$_2^-$ in CLL cells and that combination of As$_2$O$_3$ with 2-ME causes an increased accumulation of superoxide and a significant enhancement of apoptosis in CLL cells (42). For instance, fresh CLL cells from 17 patients incubated in vitro with $10 \mu$M 2-ME and 1 $\mu$g/ml As$_2$O$_3$ caused an additive or more than additive accumulation of O$_2^-$ in the CLL cells, and a greater than additive cell killing. Together, these observations led us to investigate the effect of As$_2$O$_3$ on mitochondrial respiratory chain and ROS generation in the present study. Our finding that arsenic trioxide increases superoxide generation in leukemia cells by interfering with mitochondrial respiration and ROS generation suggests that the mitochondrial respiration and ROS generation are important for the drug action. Third, the effective suppression of drug-induced apoptosis further suggests that ROS is the critical mediator of drug-induced apoptosis.

The ability of arsenic trioxide to interfere with the mitochondrial respiration function and increase ROS generation suggests that this compound may be useful for combination with 2-ME and other ROS-generating agents such as doxorubicin, bleomycin, and cisplatin to enhance their anticancer activity. Because all these agents are currently used in the clinical treatment of cancer or are under clinical investigation, the application of such biochemical mechanism-based combination strategy is of therapeutic significance, and warrants further consideration and evaluation in vivo.

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Inhibition of Mitochondrial Respiration: A NOVEL STRATEGY TO ENHANCE DRUG-INDUCED APOPTOSIS IN HUMAN LEUKEMIA CELLS BY A REACTIVE OXYGEN SPECIES-MEDIATED MECHANISM

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