Curcumin Stimulates Reactive Oxygen Species Production and Potentiates Apoptosis Induction by the Antitumor Drugs Arsenic Trioxide and Lonidamine in Human Myeloid Leukemia Cell Lines

Yolanda Sánchez, Gloria P. Simón, Eva Calviño, Elena de Blas, and Patricio Aller
Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain

Received March 22, 2010; accepted July 1, 2010

ABSTRACT

Arsenic trioxide (ATO, Trisenox) is an important antileukemic drug, but its efficacy is frequently low when used as a single agent. Here, we demonstrate that the apoptotic action of ATO is greatly increased when combined with subcytotoxic curcumin concentrations in U937 and HL60 human acute myeloid leukemia cells, and with lower efficacy in K562 chronic myelogenous leukemia cells. Curcumin exerts similar cooperative effect with the mitochondria-targeting drug lonidamine, whereas the response is negligible in combination with the DNA-targeting drug cisplatin. Curcumin plus ATO or lonidamine stimulates typical events of the mitochondrial executioner pathway (Bax and Bid activation, cytochrome c release, X-linked inhibitor of apoptosis downregulation, and caspase-9/-3 activation) and causes mitochondrial transmembrane potential dissipation, which nevertheless represents a late event in the apoptotic response. Curcumin increases anion superoxide production, and its proapoptotic action in combination with ATO and lonidamine is mimicked by pro-oxidant agents (2-methoxyestradiol and H₂O₂) and prevented by antioxidant agents [Mn(III)tetrakis(4-benzoic acid)porphyrin chloride and N-acetyl-L-cysteine]. Within the assayed time period (16–24 h), curcumin does not significantly modify p38-mitogen-activated protein kinase and c-Jun NH₂-terminal kinase phosphorylation/activation or nuclear factor-κB activity, but it greatly stimulates extracellular signal-regulated kinase (ERK) phosphorylation, and decreases Akt phosphorylation. Experiments using mitogen-activated protein kinase kinase/ERK inhibitors [2-amino-3-methoxyflavone (PD98059) and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminoethylthio]butadiene (U0126)] and phosphatidylinositol 3-kinase inhibitor (4-morpholino)-8-phenyl-1-benzopyran-4-one (LY294002) indicate that ERK activation does not mediate and even restrains apoptosis potentiation, whereas Akt down-regulation facilitates apoptosis generation. In summary, cotreatment with curcumin may represent a useful manner of increasing the efficacy of ATO and lonidamine as antitumor drugs in myeloid leukemia cells.

Introduction

Arsenic trioxide (ATO, Trisenox) is an efficacious, clinically established agent for the treatment of relapsed and refractory acute promyelocytic leukemia (APL) (Wang and Chen, 2008). At low, physiologically tolerable concentrations (0.25–2 μM in plasma), ATO causes APL cytoreduction by inducing terminal differentiation or apoptosis (Miller et al., 2002). Albeit with lower efficacy, ATO also causes apoptosis in other types of leukemia and solid tumor-derived cells, which opens the possibility of extending the therapeutic application of the drug (Amadori et al., 2005). Nonetheless, the relatively low sensitivity of most...
tumor cells to ATO would require the generation of sensitizing strategies, to increase drug efficacy and reduce dosage to clinically achievable concentrations. It is important to note that ATO is an oxidant-sensitive agent, in such a manner that drug toxicity is exacerbated under conditions of moderate oxidative stress, namely, decreased intracellular reduced glutathione (GSH) content (Dai et al., 1999), increased reactive oxygen species (ROS) accumulation (Yi et al., 2002), or both. Another aspect of interest is the high intrinsic oxidative stress of most cancer cells (Lau et al., 2008), which makes them more vulnerable to pro-oxidant treatments than their normal counterparts. For this reason, agents that generate a moderate oxidant environment may offer potential therapeutic opportunities.

Curcumin, a hydrophobic polyphenol derived from the rhizome of turmeric (Curcuma longa L.), is a safe, well tolerated, and an efficacious chemopreventive agent, mainly because of its antioxidant and anti-inflammatory properties (Anand et al., 2007). Nonetheless, curcumin also may induce apoptosis in tumor cells, potentiate apoptosis induction by classical chemotherapeutic drugs, or both, supporting its potential use in anticancer therapies (Limtrakul, 2007; Reuter et al., 2008). In fact, in spite of its poor stability and bioavailability (Anand et al., 2007; Basile et al., 2009), the antioxidant efficacy of curcumin has been proved in animal models and phase I clinical trials with multiple types of cancer (Anand et al., 2008). As with other plant-derived polyphenols, the pro-apoptotic action of curcumin has been largely attributed to the inhibition of defensive signaling pathways such as PI3K/Akt and NF-κB (Lin, 2007; Reuter et al., 2008), but other mechanisms may be equally important. For example, in spite of its normal antioxidant capacity, curcumin may per se stimulate ROS production (Woo et al., 2003; Chan et al., 2006) or potentiate ROS generation by other agents (Javad et al., 2008; Kikuchi et al., 2010) in different tumor cell models. Moreover, curcumin may oxidize thiol residues at the mitochondrial transition pore (mPTP), causing mitochondria dysfunction and cell death (Ligeret et al., 2004).

Previous studies indicated that curcumin attenuates oxidative stress generation and other toxic effects of arsenic in nontumor cells in culture (e.g., peripheral blood lymphocytes and Chinese hamster fibroblasts) and animal models (Mukherjee et al., 2007; Roy et al., 2008; Yadav et al., 2009, among others). Nonetheless, we reported that another phenolic agent, the soy isoflavone genistein, potentiates ATO-provoked apoptosis in leukemia cell models but not in normal peripheral blood lymphocytes and that this effect is mediated by genistein-provoked ROS overproduction and activation of ROS-inducible protein kinases (Sánchez et al., 2008). Thus, in the present work we analyze the capacity of curcumin to modulate ATO-provoked apoptosis in human U937 and HL60 acute myeloid leukemia (AML) and K562 chronic myelogenous leukemia (CML) cells. These cell lines are considered as relatively resistant to ATO in comparison with the highly sensitive APL cells (Jing et al., 1999) and hence are appropriate to study possible cooperative effects. For comparison, the action of curcumin was assayed in combination with two other anticancer drugs, cisplatin and lonidamine. Cisplatin is a DNA-targeting drug, whereas lonidamine and ATO are classified as mitochondria-targeting drugs, capable of binding the adenine nucleotide translocator at the mPTP, causing pore opening and mitochondria dysfunction (Solary et al., 2003; Fantin and Leder, 2006). The obtained results indicate that curcumin greatly cooperates with ATO and lonidamine to induce apoptosis, whereas the response is negligible in the case of cisplatin. The pro-apoptotic action of curcumin is mediated by ROS overproduction and, at least in the case of ATO, also by Akt down-regulation.

Materials and Methods

Reagents and Antibodies. All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Monochlorobimane and MitoProbe DiIC$_3$(5) assay kit for flow cytometry were obtained from Molecular Probes (Eugene, OR). Dihydroethidium (DHE, presented as a 5 mM solution in dimethyl sulfoxide) was obtained from Invitrogen. 4,6-Diamino-2-phenylindole (DAPI) was obtained from Calbiochem (Darmstadt, Germany). Recombinant human tumor necrosis factor-α was obtained from PeproTech EC (London, UK). The kinase inhibitors PD98059, U0126, and LY294002; the caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp(OMe)-CH$_2$F (z-Val-fmk); and Mn(III)tetrakis(4-benzoic acid)porphyrin chloro (MnTBAP) were obtained from Calbiochem (Darmstadt, Germany). Rabbit anti-human p38-MAPK, phospho-p38-MAPK (Thr180/Tyr182), stress-activated protein kinase/JNK, phospho-stress-activated protein kinase/JNKThr183/Tyr185, p44/42 MAPK, phospho-p44/p42 MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), and cleaved caspase-3 (Asp175) polyclonal antibodies (pAbs) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Mouse anti-pig c-rosylc c monoclonal antibody (mAb) clone H8.2C12 was obtained from BD Biosciences Pharmingen (San Diego, CA). Mouse anti-human Bcl-2 (100 MAb; rabbit anti-human Bax (N-20), anti-human caspase-9 p35 (H-170), anti-human NF-κB p65(A), and anti-human Sam 68 (C-20) pAbs; and goat anti-human Bid (C-20) pAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-XIAP (clone 2F1) mAb was obtained from MBL International (Woburn, MA). Peroxidase-conjugated immunoglobulin G antibodies were obtained from Dako Diagnostics, S.A. (Barcelona, Spain). All other reagents and antibodies were from Sigma (Madrid, Spain).

Cells and Treatments. U937 and HL60 human AML cells and K562 human CML cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated bovine serum and antibiotics, in a humidified 5% CO$_2$ atmosphere at 37°C. Sixteen to 24 h before the initiation of the treatment, the cell concentration was adjusted to approximately 10$^5$ cells/ml. Nonetheless, to prevent the cultures from reaching plateau densities or nutrient exhaustion in long-term experiments (72 h), at the second day of treatment they were supplemented with an equal volume of fresh culture medium containing the corresponding drugs.

Stock solutions of curcumin, cyclosporine A (CsA), 2-methoxyestradiol (2-ME), PD98059, U0126, LY294002 (20 MAb each), genistein (50 M), z-Val-fmk (25 M), monochlorobimane (200 M), lonidamine (100 M), and N-acetyl-l-cysteine (NAC; 3 M) were prepared in dimethyl sulfoxide. A stock solution of cis-platinum (II)-diamine dichloride (cisplatin, CDDP; 3.3 M) was prepared in distilled water. MnTBAP was dissolved at 100 mM in aqueous base. 3(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was dissolved at 5 mg/ml in phosphate-buffered saline (PBS). All these solutions were stored at −20°C. Stock solutions of DAPI (10 µg/ml) and propidium iodide (PI; 1 mg/ml) were prepared in PBS. l,l-Buthionine-(S,R)-sulfoximine (BSO) was dissolved at 50 mM in distilled water. ATO was initially dissolved in a small amount of N NaOH, and then diluted with PBS to give a final concentration of 10 µM. These solutions were stored at −4°C.

Flow Cytometry. The analysis of samples was carried out using an FC-500 cytometer analyzer (Beckman Coulter, Hialeah, FL) equipped with two air-cooled argon lasers tuned to 488 nm (for DHE,
PI, and annexin V-FITC) and 630 nm [for DilC<sub>5</sub>(5)]. The specific fluorescence signal corresponding to annexin V-FITC was collected with a 525-nm bandpass filter, the signal corresponding to DilC<sub>5</sub>(5) with a 675-nm bandpass filter, and the signals corresponding to DHE and PI with a 620-nm bandpass filter. In total, 10<sup>4</sup> cells were scored in each assay.

**Measurement of Cell Viability and Apoptosis.** The relative number of viable cells in the culture was determined using the MTT colorimetric assay. With this aim, the cells were centrifuged, resuspended in 1 mL of RPMI 1640 medium without red phenol containing 0.5 mg/mL MTT, distributed in quadruplicate in 96-well plates, and incubated for 2 to 3 h at 37°C, after which SDS was added to a final concentration of 2% to dissolve the formazan crystals. Dye absorbance was measured by spectrometry at 595 nm using an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Under these conditions, a decrease in absorbance may reflect inhibition of cell proliferation, increase in cell death, or the sum of both factors. Alternatively or complementarily, cells were incubated with trypan blue, and the number of viable cells (excluding the dye) was determined by microscopy.

Distinctive characteristics of apoptotic cells were chromatin condensation/fragmentation, reduction in DNA content (sub-G<sub>1</sub>), and phosphatidylserine translocation from the inner to the outer layer of the plasma membrane. Chromatin condensation was determined by cell permeabilization followed by DAPI staining and microscopy, and reduction in DNA content was determined by cell permeabilization followed by PI staining and flow cytometry. The latter method also was used to analyze cell cycle phase distribution. A detailed description of these procedures was presented in a previous publication (Troyano et al., 2003) and is omitted here. Phosphatidylserine translocation was estimated by measuring cell surface binding of annexin V-FITC by means of flow cytometry, using an ApoAlert annexin V-FITC kit, (Clontech, Mountain View, CA), following the indications of the manufacturer. Cells treated with curcumin but unlabeled with annexin V were included as controls to measure basal drug autofluorescence. As a routine, free penetration of trypan blue into nonpermeabilized cells was examined as an indication of loss of plasma membrane integrity (primary or secondary necrosis).

**Determination of Mitochondrial Transmembrane Potential.** At the end of treatments, the cells were washed twice with PBS and incubated for 30 min at 37°C with RPMI 1640 medium containing 1 mM DilC<sub>5</sub>(5), after which they were washed twice in ice-cold RPMI 1640 medium containing 1% fetal bovine serum, resuspended in ice-cold RPMI 1640 medium, and the fluorescence was analyzed by flow cytometry. Cells incubated with the depolarizing agent carbonyl cyanide 3-chlorophenylhydrazone (50 μM) were used as an internal control.

**Determination of ROS Production.** At the end of treatments, the cells were washed twice with PBS and incubated for 30 min at 37°C with RPMI 1640 medium containing 5 μM DHE, a fluorescent probe that specifically detects anion superoxide production. The cells were then washed twice with cold PBS and resuspended in cold RPMI 1640 medium, and the fluorescence analyzed by flow cytometry. Under these conditions, residual curcumin-derived autofluorescence was negligible, as determined using DHE-unlabeled cells. Other commonly used fluorescent probes such as dichlorodihydrofluorescein diacetate were avoided, because residual curcumin autofluorescence at the required wavelength (525-nm bandpass filter) was still evident.

**Measurement of GSH Level.** The total intracellular GSH content was determined by fluorometry after cell loading with monochlorobimane, following a previously described procedure (Troyano et al., 2001).

**Cell Fractionation and Immunoblot Assays.** To obtain total cellular protein extracts, cells were collected by centrifugation, washed with PBS, and lysed for 20 min at 4°C in a buffer consisting of 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, 10% (v/v) glycerol, and 1% (v/v) Nonidet P-40, supplemented with a protease inhibitor cocktail, 1 mM sodium orthovanadate, and 10 mM NaF. After centrifugation at 10,000g for 15 min at 4°C, the supernatants were collected. To obtain mitochondrial extracts, aimed at determining Bax translocation to mitochondria, cells were homogenized by repeatedly passing them throughout a 25-gauge needle. The homogenate was first centrifuged at 1000g for 10 min, and the supernatant was centrifuged again at 10,000g for 20 min to obtain the mitochondrial fraction, following the previously described procedure (Troyano et al., 2003). Cytosolic extracts, aimed at determining cytochrome c release from mitochondria, and nuclear extracts, aimed at determining p53 NF-kB translocation, were prepared as described previously (Sánchez et al., 2008, and references therein). Fractions of total, mitochondrial, cytosolic, or nuclear extracts, containing equal protein amounts, were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected, as described previously (Galán et al., 2000).

**Electrophoretic Mobility Gel Shift Assays.** All experimental conditions, including nuclear extract preparations, oligoprobe preparation, radioactive labeling, binding reactions, and electrophoretic separation, were as described previously (Sánchez et al., 2008, and references therein).

**Statistical Analysis.** When convenient, the significance of differences between experimental conditions was examined using the Student’s t test, and positive differences are indicated by asterisks (*, p < 0.05, **, p < 0.01, and ***, p < 0.001).

**Results**

**Apoptosis Generation.** Figure 1 shows the capacity ATO (2–6 μM) and curcumin (from 5 to 20 μM), used alone and in combination, to decrease viability, induce apoptosis, and affect growth cycle progression in U937 human AML cells. As indicated in Fig. 1A, 2 μM ATO and 5 and 7.5 μM curcumin caused minimal (less than 10%) apoptosis, whereas moderate apoptosis (20–40%) was obtained with 4 to 6 μM ATO and 10 to 20 μM curcumin, as measured at 24 h of treatment by chromatin condensation/fragmentation. Thus, the concentrations of 2 μM ATO and 7.5 μM curcumin were selected for combined treatments. When used in combination, curcumin and ATO cooperated in more than an additive manner to induce apoptosis, as determined by chromatin fragmentation (Fig. 1A), frequency of cells with sub-G<sub>1</sub> DNA content (Fig. 1D), and annexin V binding at the cell surface (Fig. 1E), and they also cooperated to reduce cell viability, as determined by the MTT assay (Fig. 1B). Time course assays indicated that significant apoptosis started to be detected at 16 h of treatment in the combined treatments (Fig. 1C). The treatments caused minimal alterations in cell cycle distribution, except for a slight decrease in the G<sub>2</sub>/M compartment in curcumin-treated cultures (Fig. 1D). Finally, cotreatment with the pan-caspase inhibitor z-VAD-fmk greatly reduced the frequency of cells with fragmented chromatin in curcumin plus ATO-treated cultures (Fig. 5C), which corroborates that the observed cell death is bona fide caspase-dependent apoptosis.

For comparative purposes, we examined the action of curcumin and ATO, alone and in combination, in HL60 AML and K562 CML cell lines. The effects of the treatments on viability, cycle phase distribution, and apoptosis generation in HL60 cells were similar to those in U937 cells (Fig. 2A; data not shown). In contrast, a higher ATO concentration (10 μM) was required to obtain significant apoptosis as well as cooperation between ATO and curcumin in K562 cells at 24 h of treatment (Fig. 2A).

The relative resistance of this cell line against apoptosis was
not compensated by a necrotic response, which at 24 h remained below 6%, similarly to untreated cells, as revealed by trypan blue assays (data not shown). Nonetheless lower ATO concentrations (5 μM) affected cell proliferation, as manifested by cell accumulation at G2/M (Fig. 2B), with a consequent decrease in the number of viable cells, as indicated by MTT assay (Fig. 2C). Of note, ATO-provoked cell cycle disturbance was a characteristic of K562 cells, because treatment of U937 and HL60 cells with 4 and 6 μM ATO, which as indicated above markedly
induced apoptosis (Fig. 1A), did not significantly affect cycle phase distribution within the 24-h time period (data not shown).

Finally, we analyzed the capacity of curcumin to modulate apoptosis induction by lonidamine (25–100 μM) and cisplatin (1–4 μM) in U937 cells. Generally, 25 and 50 μM lonidamine and 1 to 2 μM cisplatin caused minimal (less than 10%) apoptosis, whereas moderate apoptosis (approximately 20–25%) was obtained with 100 μM lonidamine and 4 μM cisplatin, at 24 h of treatment (Fig. 3A). Nonetheless, the toxicity of different lonidamine stocks varied considerably; hence, different concentrations had to be adopted for mechanistic assays. When used in combination, curcumin and lonidamine greatly cooperated to induce apoptosis, as manifested by chromatin fragmentation (Fig. 3A), sub-G₀ DNA content (Fig. 3C), and cell surface annexin V binding (Fig. 3D), and to reduce cell viability, as manifested by MTT assay (Fig. 3B). Alternatively, the combination of curcumin plus cisplatin produced lower (less than additive) effects on apoptosis. Of note, the low apoptotic response was not compensated by the generations of necrosis, which was negligible (less than 10%), as revealed by the absence of trypan blue uptake (data not shown) and PI uptake in the annexin V/PI assays (Fig. 3D). Nonetheless, in accordance to its property as a DNA-damaging agent, cisplatin affected cell cycle transition throughout the S and G₂/M phases (Fig. 3C), which may explain the reduction in the total number of viable cells, as revealed by the MTT assay (Fig. 3B). This cell cycle disturbance was attenuated by cotreatment with curcumin (Fig. 3C). In another group of experiments, U937 cells were subjected to long-term treatments (72 h) with 2 μM cisplatin. Under these conditions, the drug caused a great decrease in cell number, cell accumulation at G₂/M, cell hypertrophy, apoptosis, and necrosis (probably “secondary”, apoptosis-derived necrosis). Cotreatment with 2 and 4 μM curcumin, which per se reduced cell proliferation without causing cell death, did not potentiate but instead slightly attenuated cisplatin-provoked apoptosis (Table 1).

Mitochondria Regulatory Events. Because ATO and lonidamine are mitochondria-targeting drugs, we analyzed the behavior of proteins that are known to regulate apoptosis execution along the intrinsic (mitochondrial) pathway. Determinations were carried out at 16 h of treatment, the time at which apoptosis was first detected (Fig. 1C). Some results, obtained by immunoblot assays, are represented in Fig. 4. We observed the following. 1) Treatments caused little effect on total Bcl-2 and Bax expression, except for a slight decrease in Bcl-2 in the curcumin plus lonidamine treatment. 2) Nonetheless, treatments caused proapoptotic BID and Bax activation, as manifested by the decrease in the amount of the 23-kDa BID proform (as an indirect evidence of protein truncation/activation) and Bax translocation to the mitochondrial fraction (with concomitant decrease in the cytosolic fraction). 3) Treatments stimulated cytochrome c release from mitochondria to the cytosol, required for apoptosisosome formation and activation, as demonstrated using cytosolic protein extracts. 4) Treatments also down-regulated the expression of XIAP, which may relieve caspases from the inhibitory action exerted by this protein. 5) As a consequence, the treatments caused the truncation/activation of caspase-9, the initiator caspase in the mitochondrial pathway, and of the executioner caspase-3. In general, maximal alterations were produced by drug combinations (curcumin plus either lonidamine or ATO), which is consistent with the higher rate of apoptosis generation (Figs. 1 and 3).

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**Fig. 3.** Apoptosis induction and cell cycle effects caused by lonidamine, cisplatin, and curcumin in U937 cells. A, frequency of apoptosis, as determined by chromatin condensation/fragmentation, in U937 cell cultures treated with the indicated concentrations of lonidamine (Lon; top bar chart) and cisplatin (CDDP; bottom bar chart), alone and in combination with curcumin. B, changes in cell viability, as determined by MTT assay. C, cell-cycle distribution and frequency of apoptotic cells. D, frequency of cells exhibiting phosphatidylserine translocation, as measured by cell surface annexin V binding (green fluorescence) and PI uptake (red fluorescence). Except when otherwise indicated, curcumin was used at 7.5 μM, Lon at 50 μM, and CDDP at 4 μM. All determinations were carried out at 24 h of treatment. All other conditions were as described in Fig. 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell No.</th>
<th>Apoptotic Cells</th>
<th>Cells at G₂/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>4 ± 1</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Curcumin, 2 μM</td>
<td>85 ± 9</td>
<td>5 ± 2</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Curcumin, 4 μM</td>
<td>65 ± 6</td>
<td>7 ± 2</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>CDDP, 2 μM</td>
<td>15 ± 3</td>
<td>43 ± 6</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>CDDP, 2 μM + curcumin, 2 μM</td>
<td>17 ± 2</td>
<td>28 ± 4</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>CDDP, 2 μM + curcumin, 4 μM</td>
<td>15 ± 5</td>
<td>35 ± 6</td>
<td>45 ± 7</td>
</tr>
</tbody>
</table>

a Cells were seeded at 10⁵ cells/ml. The approximate final concentration in control was 17 ± 10² cells/ml.

b Measured by chromatin condensation/fragmentation.

c Relative to the total number of cells at G₀ + S + G₂/M (excluding cells at sub-G₀).

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In addition, we analyzed the capacity of the treatments to cause mitochondrial membrane potential (ΔΨm) dissipation, as a manifestation of mitochondrial dysfunction. It was observed that curcumin plus ATO or lonidamine elicited the appearance of a subpopulation of cells with reduced ΔΨm, as indicated by the reduction in DilC1(5)-derived fluorescence (Fig. 5A). Nonetheless the importance of this event as a regulator of apoptosis was unclear. In fact, ΔΨm dissipation was only detectable at 16 h of treatment (Fig. 5B), which coincided with the initiation of apoptosis execution (Fig. 1C). Moreover, ΔΨm reduction appeared as a caspase-dependent response, which was prevented by the pan-caspase inhibitor z-VAD-fmk (Fig. 5C, top bar chart). In addition, cotreatment with CsA (2–7 μM), an agent that inhibits mPTP by binding to cyclophilin D, failed to prevent ΔΨm dissipation as well as apoptosis execution (Fig. 5C; data not shown).

**ROS Production and Effect of Antioxidant Agents.**

As mentioned, ATO toxicity is increased under conditions of intracellular GSH depletion or ROS overaccumulation. It was observed that 7.5 μM curcumin did not decrease and even slightly augmented GSH content, as measured by monochlorobimane derivatization. This contrasts with the depletion caused by the typical GSH synthesis inhibitor BSO, included as a control (Fig. 6A). In contrast, 7.5 μM curcumin caused an early and slight, but statistically significant, increase in ROS accumulation, as measured using the anion superoxide-sensitive probe DHE (Fig. 6B). The importance of ROS production for curcumin-provoked apoptosis potentiation was proved using the ROS scavengers NAC and MnTBAP, a cell-permeable superoxide dismutase mimetic agent. It was observed that cotreatment with the antioxidants greatly attenuated apoptosis generation (Fig. 6C), ΔΨm dissipation (Fig. 6D) and cytochrome c release (Fig. 6E) by curcumin plus either ATO or lonidamine. NAC and MnTBP also attenuated apoptosis generation by curcumin alone, when used at a toxic concentration (20 μM) (Fig. 6C). Finally, to corroborate the importance of the oxidant environment for apoptosis potentiation, cells were coincubated with ATO or lonidamine plus a low concentration of H2O2, a paradigmatic pro-oxidant agent, or 2-ME, which is known to increase intracellular anion superoxide accumulation by inhibiting superoxide dismutase activity (Huang et al., 2000). The results in Fig. 6F indicated that H2O2 and 2-ME cooperate with...
ATO and lonidamine in more than additive manner to cause apoptosis, with better response for H$_2$O$_2$ in the case of ATO, and for 2-ME in the case of lonidamine. Alternatively, the pro-oxidant agents either did not affect cisplatin toxicity (2-ME) or another study (Fernández et al., 2004), either did not modify (H$_2$O$_2$), a result that parallels oxidant agents either did not affect cisplatin toxicity (2-ME) or the presence of NAC (+ NAC) or MnTBAP (+ MnTBAP). F, frequency of apoptosis upon treatment with ATO, lonidamine, and cisplatin, alone (−) or together with H$_2$O$_2$ (+ H$_2$O$_2$) or 2-methoxyestradiol (+ 2-ME). Symbols indicate that the values in the combined treatments (ATO + H$_2$O$_2$, Lon + H$_2$O$_2$, ATO + 2-ME, and Lon + 2-ME) is equivalent (#) or significantly higher (asterisks) than the sum of values in the corresponding single treatments. Curcumin was used at 7.5 μM (except when otherwise indicated), ATO at 2 μM, lonidamine at 100 μM, cisplatin at 4 μM, NAC at 10 mM, MnTBAP at 100 μM, H$_2$O$_2$ at 40 μM, and 2-ME at 0.5 μM. NAC and MnTBAP were applied 2 h before the other agents. Results in D and E were obtained at 16 h of treatment and in C and F at 24 h of treatment. All other conditions were as described in Figs. 1 and 3.

Protein Kinase Modulation and Effects of Protein Kinase Inhibitors. MAPK p38 has been characterized as a kinase responsive to moderate oxidative stress (Kurata, 2000). In addition, other studies reported that curcumin stimulates ROS generation and JNK activation in osteoblast cells (Chan et al., 2006), potentiates ROS-dependent ERK activation and lethality in irradiated human cervical tumor cells (Javvadi et al., 2008), and causes ROS-mediated Akt inactivation in human renal cells (Woo et al., 2003). Thus, we wanted to measure the phosphorylation/activation state of these kinases in U937 cells treated with curcumin alone and in combination with ATO and lonidamine. The results were as follows. 1) The treatments failed to significantly affect p38-MAPK and JNK phosphorylation (Supplemental Fig. 1). 2) In contrast, curcumin alone or with ATO, greatly stimulated ERK phosphorylation/activation at 14 to 24 h, and this response was corroborated (24 h) using lonidamine plus curcumin (Fig. 7A). Nonetheless ERK activation did not explain apoptosis potentiation in our studies. In fact, the MEK/ERK inhibitors PD98059 (20 μM) and U0126 (2.5 μM), which successfully prevented kinase activation in U937 cells in another study (Fernández et al., 2004), either did not modify

**Fig. 6.** Changes in intracellular GSH and ROS levels, and effect of antioxidants or pro-oxidants. A, changes in intracellular GSH concentration, as indicated by monochlorobimane derivatization, in cells treated for the indicated times with curcumin or 1 mM BSO, included as a control. The results are represented in relation to untreated (Cont) cells (approximate GSH content; 9.5 nmol/10$^6$ cells), which received the arbitrary value of 1.0. B, intracellular ROS accumulation, as indicated by DHE-derived fluorescence, in U937 cells treated for the indicated times with curcumin, ATO, and lonidamine. The results are represented in relation to the untreated cells, which received the arbitrary value of 1.0. Asterisks indicate significant differences in relation to control. An example of cell distribution in untreated (Cont) and curcumin-treated cultures is indicated at the bottom. The vertical dotted line indicates the main value in control, to better discern the displacement caused by curcumin. C to E, frequency of apoptotic cells (C), ΔΨm dissipation (D), and cytochrome c release to the cytosol (E) upon treatment with 20 μM curcumin alone or with the combinations of ATO plus curcumin or lonidamine plus curcumin, in the absence (−) or the presence of NAC (+ NAC) or MnTBAP (+ MnTBAP). F, frequency of apoptosis upon treatment with ATO, lonidamine, and cisplatin, alone (−) or together with H$_2$O$_2$ (+ H$_2$O$_2$) or 2-methoxyestradiol (+ 2-ME). Symbols indicate that the values in the combined treatments (ATO + H$_2$O$_2$, Lon + H$_2$O$_2$, ATO + 2-ME, and Lon + 2-ME) is equivalent (#) or significantly higher (asterisks) than the sum of values in the corresponding single treatments. Curcumin was used at 7.5 μM (except when otherwise indicated), ATO at 2 μM, lonidamine at 100 μM, cisplatin at 4 μM, NAC at 10 mM, MnTBAP at 100 μM, H$_2$O$_2$ at 40 μM, and 2-ME at 0.5 μM. NAC and MnTBAP were applied 2 h before the other agents. Results in D and E were obtained at 16 h of treatment and in C and F at 24 h of treatment. All other conditions were as described in Figs. 1 and 3.

Fig. 7. ERK modulation and effect of MEK/ERK inhibitors. A, relative levels of total (T) and phosphorylated (P) ERK, upon treatment for the indicated times with curcumin, ATO plus curcumin, or ATO plus lonidamine, as determined by immunoblot assays. B, frequency of apoptotic cells at 24 h of treatment with ATO or lonidamine alone (−), with ATO or lonidamine plus curcumin (+ Cur), and with ATO or lonidamine plus curcumin in the presence of the MEK/ERK inhibitors PD98059 (+ PD+Cur) or U0126 (+ U+Cur). Asterisks indicate significant differences in relation to control. C, cell cycle distribution and frequency of apoptotic (sub-G$_1$) cells at 24 h of treatment with the indicated agents. The MEK/ERK inhibitors were applied 1 h in advance to the other agents. Curcumin was used at 7.5 μM, ATO at 2 μM, PD98049 at 30 μM, and U0126 at 2.5 μM. All other conditions were as described in Fig. 1.
applied in advance to the other drugs. All other conditions were as described in Fig. 1. (sub-G1) cells at 24 h of treatment with the indicated agents. ATO was used at 2 
LY (right bar chart). Asterisks indicate significant differences in relation to control. C, cell cycle distribution and frequency of apoptotic

sensitivity of ATO, lonidamine, and cisplatin to the pro-oxidant

polyphenol was combined with cisplatin. There are several

contrast, the cooperative response was negligible when the

intensity of bands in relation to the control (which received the arbitrary value of 1), as determined by densitometry. B, frequency of apoptotic cells

with the PI3K inhibitor LY294002 (LY), and with ATO or lonidamine, either alone (–) or in combination with LY294002 (+LY) (left bar chart); or with curcumin, ATO plus curcumin, and lonidamine plus curcumin, either alone (+Cur) or in combination with LY294002 (+Cur+LY) (right bar chart). Asterisks indicate significant differences in relation to control. C, cell cycle distribution and frequency of apoptotic

(sub-G1) cells at 24 h of treatment with the indicated agents. ATO was used at 2 µM, curcumin at 7.5 µM, and LY294002 at 30 µM. LY294002 was applied 1 h in advance to the other drugs. All other conditions were as described in Fig. 1.

Discussion

The results in this work indicate that cotreatment with subcytotoxic curcumin concentrations greatly increases the apoptotic efficacy of ATO and lonidamine in U937 and HL60 AML cells and, albeit with lower intensity, K562 CML cells. To our knowledge, this is first documented observation indicating ATO and lonidamine sensitization by curcumin, because as indicated above, all preceding publications only reported protection by curcumin against arsenic toxicity. In contrast, the cooperative response was negligible when the polyphenol was combined with cisplatin. There are several mechanisms that could explain the drug-dependent, differential efficacy of curcumin. One of them is the unequal sensitivity of ATO, lonidamine, and cisplatin to the pro-oxidant environment, as discussed below. Another reason is that although ATO and lonidamine exerted minimal effects on cell cycle distribution in U937 and HL60 cells, the DNA-damaging agent cisplatin delayed or blocked cell transition throughout S and G2 with concomitant decrease in the size of the G1 subpopulation. This is important because cells at G2/M are more resistant to curcumin than cells traversing the G1 phase (Wang et al., 2008). In good agreement with this hypothesis, ATO caused G2/M arrest in K562 cells, and these cells were relatively resistant to apoptosis induction by ATO plus curcumin. Nonetheless, other explanations are possible in this particular case, such as the high intrinsic resistance of K562 cells conferred by the p210BCR/ABL kinase. Regardless, it must be noted that curcumin was reported to efficaciously potentiate cisplatin-provoked apoptosis in other cell types (Notarbartolo et al., 2005; Chanvorachote et al., 2009), indicating that treatment effects are not only drug-specific but also cell type-specific.

In agreement with the properties of ATO and lonidamine as mitochondria-targeting drugs, the treatments activated apoptosis through the mitochondrial executioner pathway, as evidenced by Bax- and Bid-regulated cytochrome c release, down-modulation of the caspase inhibitory XIAP protein, and the resulting activation of the caspase-9/ caspase-3 pathway. In addition the treatments caused ΔΨm disruption, but the response pattern was not as initially expected. In fact, ΔΨm dissipation represented a late, caspase-dependent (z-VAD-inhibitable) event, occurring at the same time as other apoptotic features (e.g., caspase-3 activation, chromatin condensation/fragmentation). Moreover, cotreatment with the mPTP inhibitor CsA, which is supposed to block mPTP opening, failed to prevent ΔΨm dissipation and apoptosis generation. Nonetheless these observations must be considered with caution, because drug responses are not necessarily equivalent when examined at the subcellular level (e.g., PTP opening in isolated mitochondria) and in entire cells.
where multiple parameters may be affected. For example, it was reported that CsA triggers ROS production in several cell models (Navarro-Antolín et al., 2007, and references therein), and ROS are determinants for ATO and lonidamine toxicity, as mentioned. In addition, the ATO concentration used by us (2 µM) is within the range of clinical useful concentrations but is much lower than those currently used to analyze mPTP induction. Finally, lonidamine may inhibit mitochondria-bound hexokinase, affecting glucose metabolism in tumor cells (Fantis and Leder, 2006). Hence, its proapoptotic action might be mainly the consequence of ATP depletion instead of direct mPTP induction. These possibilities are currently under study.

In addition, our results indicate that ATO as well as lonidamine are oxidant-sensitive agents, as proved by the increased toxicity when combined with low H₂O₂ concentrations or the superoxide dismutase inhibitor 2-ME. In spite of their normal protective, antioxidant action, acute exposure to some polyphenols may stimulate mitochondrial (Salvi et al., 2002) and extramitochondrial (Galati et al., 2002) ROS production. In this regard, Kikuchi et al. (2010) recently indicated that curcumin potentiated NADPH-derived anion superoxide generation by all-trans-retinoic acid in U937 cells, although treatment with curcumin alone was ineffective. However, our present results indicate that curcumin alone suffices to slightly stimulate anion superoxide generation in this cell line, although the exact mechanism was not investigated. ROS generation by a relatively high curcumin concentration (20 µM) might per se suffice to trigger apoptosis, whereas the slight ROS production caused by a lower (7.5 µM) concentration, being itself innocuous, may increase ATO and lonidamine toxicity, as proved by the protective action of the antioxidants NAC and MnTBAP. Moreover, as mentioned, this mechanism may provide an alternative or complementary explanation for the inability of curcumin to potentiate cisplatin-provoked apoptosis. In fact, under the conditions used here, cisplatin was less sensitive to the prooxidant environment, as revealed by cotreatment with H₂O₂ or 2-ME.

Finally, we analyzed the behavior of some protein kinases the expression of which might undergo ROS-dependent alterations in curcumin-treated cells. Curcumin caused ERK phosphorylation/activation, but this response was not affected (curcumin plus lonidamine) or was even potentiated (curcumin plus ATO) by cotreatment with pharmacologic MEK/ERK inhibitors. This is consistent with the protective, antiapoptotic action commonly attributed to this kinase but contrasts with the proapoptotic function of curcumin-provoked ERK activation reported in another experimental model (Javvadi et al., 2008). In addition, in spite of its proapoptotic action, curcumin failed to significantly stimulate JNK and p38-MAPK activation. This latter result was unexpected, because as indicated, p38-MAPK is considered as a sensor of moderate oxidative stress, and this kinase was activated in a ROS-dependent manner by other phenolic agents in U937 cells (Sánchez et al., 2008). Nonetheless, it was reported that curcumin may cause p38-MAPK dephosphorylation via mitogen-activated protein kinase phosphatase-5 activation in prostate cells (Nnon et al., 2007). Thus, different opposite signals may probably concur to define the state of p38-MAPK activation in curcumin-treated cells. Finally, curcumin slightly caused Akt dephosphorylation/inactivation. In accordance with its role as a protective kinase, Akt inhibition may represent an additional mechanism explaining the potentiation by curcumin of ATO-provoked apoptosis, because ATO toxicity also was increased when combined with the PI3K inhibitor LY294002 instead of curcumin. The importance of Akt inhibition in lonidamine is less clear, because LY294002 only augmented the toxicity of lonidamine in combination with curcumin (which was itself sensitive to the PI3K inhibitor) but not of lonidamine alone. Whatever the case, Akt down-regulation contrasts with the apparent inability of curcumin to affect basal NF-κB activity, as measured by p65 subunit translocation and transcription factor binding. This does not exclude that curcumin might inhibit NF-κB in other experimental conditions, e.g., treatment with curcumin alone for longer treatment periods, or curcumin in combination with stressing, NF-κB-activating agents, as we showed previously by using another polyphenol (Sánchez et al., 2008).

Lonidamine is a well tolerated drug, clinically used against multiple types of solid tumors (Di Cosimo et al., 2003) and also of potential interest against some hematological malignancies (Solary et al., 2003). Nonetheless, as in the case of ATO the efficacy of this agent is normally low when used as monotherapy. The present results indicate that curcumin efficaciously cooperates with both ATO and lonidamine to induce apoptosis in acute myeloid leukemia cell models, a response which, considering the high tolerability of this polyphenol, might be of therapeutic interest. Although other mechanisms are also possible, such cooperation may be in part explained by curcumin-provoked generation of moderate oxidative stress, and at least in the case of ATO also by Akt down-regulation.

References
Curcumin Increases Arsenic Trioxide and Lonidamine Toxicity


