

# Pharmacologic inhibitors of PI3K/Akt potentiate the apoptotic action of the antileukemic drug arsenic trioxide via glutathione depletion and increased peroxide accumulation in myeloid leukemia cells

Adrián M. Ramos, Carlos Fernández, Donna Amrán, Patricia Sancho, Elena de Blas, and Patricio Aller

Treatment for 14 to 24 hours with low concentrations of arsenic trioxide ( $\text{As}_2\text{O}_3$ , 1-4  $\mu\text{M}$ ) caused apoptosis in U-937 promonocytes and other human myeloid leukemia cell lines (HL-60, NB4). This effect was potentiated by cotreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 and wortmannin, and the Akt inhibitor Akt5. However, the inhibitors did not increase the toxicity of the mitochondria-targeting drug lonidamine, and the DNA-specific drugs camptothecin and cisplatin, when used under similar experimental conditions as  $\text{As}_2\text{O}_3$ . The

potentiation of  $\text{As}_2\text{O}_3$ -provoked apoptosis involved the increased disruption of mitochondrial transmembrane potential, increased caspase-3 activation and cytochrome *c* release from mitochondria, increased Bax and Bid activation, and attenuation of 27-kDa heat shock protein (HSP27) expression; the potentiation was prevented by Bcl-2 overexpression. The PI3K/Akt inhibitors decreased the intracellular glutathione content, and caused intracellular oxidation, as measured by peroxide accumulation. Cotreatment with subcytotoxic concentrations of hydrogen

peroxide increased apoptosis induction by  $\text{As}_2\text{O}_3$ . On the other hand, the treatments did not significantly affect glutathione S-transferase  $\pi$  expression and activity. These results, which indicate that glutathione is a target of PI3K/Akt in myeloid leukemia cells, may partially explain the selective increase of  $\text{As}_2\text{O}_3$  toxicity by PI3K/Akt inhibitors, and may provide a rationale to improve the efficacy of these inhibitors as therapeutic agents. (Blood. 2005;105:4013-4020)

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## Introduction

Arsenic trioxide ( $\text{As}_2\text{O}_3$ , ATO) is a clinically effective agent in the treatment of acute promyelocytic leukemia (APL).<sup>1</sup> At physiologically tolerable concentrations (< 5  $\mu\text{M}$  in plasma),  $\text{As}_2\text{O}_3$  causes the degradation of the promyelocytic leukemia-retinoic acid receptor  $\alpha$  (PML-RAR $\alpha$ ) fusion oncoprotein, expressed in the vast majority of APLs, forcing the cells to terminal differentiation and/or apoptosis.<sup>1,2</sup> Moreover, albeit with lower efficacy,  $\text{As}_2\text{O}_3$  also induces apoptosis in tumor cells lacking PML-RAR $\alpha$ , including other types of leukemia and multiple myeloma, which opens the possibility for broader clinical application of this compound.<sup>1,2</sup> Nevertheless, to be effective, this possibility requires the elaboration of strategies to increase the apoptotic action of  $\text{As}_2\text{O}_3$  and to reduce the drug dosage to physiologically tolerable concentrations.

To date, the glutathione (GSH)-based redox system is the best known determinant of  $\text{As}_2\text{O}_3$  sensitivity.<sup>2,3</sup> Thus, the apoptotic action of  $\text{As}_2\text{O}_3$  inversely correlates with the endogenous levels of GSH or GSH-associated enzymes in different leukemia cell types<sup>4-6</sup>; and treatments that experimentally deplete or enhance the GSH content exacerbate or decrease, respectively, the  $\text{As}_2\text{O}_3$  toxicity.<sup>4,7</sup> Due to the multiplicity of functions of glutathione,<sup>8</sup> this molecule may modulate drug toxicity by different mechanisms. This includes the scavenging of reactive oxygen species (ROSs),

the production of which may be stimulated by  $\text{As}_2\text{O}_3$ ,<sup>5,9</sup> and the inactivation/detoxification of  $\text{As}_2\text{O}_3$ , either by direct binding to GSH<sup>10</sup> or through a reaction catalysed by glutathione S-transferases.<sup>11,12</sup> Hence, the modulation of intracellular GSH level appears as a promissory tool to regulate the clinical efficacy of  $\text{As}_2\text{O}_3$ .

An important aspect of apoptosis regulation is the signaling by serine/threonine kinases, a broad category of kinases that includes, among others, the mitogen-activated protein kinases (MAPKs) and the protein kinase B (PKB, Akt).<sup>13</sup> Among the 3 main members of the MAPK family in mammalian cells, the extracellular signal-regulated kinases (ERK1/2) are associated to mitogenesis, and as such are generally considered as antiapoptotic.<sup>13,14</sup> In a similar manner, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is considered as a critical survival-signaling pathway.<sup>15,16</sup> Akt-mediated phosphorylation may alter the activity of proteins such as caspase-9, some Bcl-2 family members, and nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and other transcription factors, which trigger or restrain apoptosis; and PI3K/Akt deregulation may contribute to tumorigenesis, metastasis, and resistance to chemotherapy.<sup>15,16</sup> For this reason, the PI3K/Akt signaling pathway represents a promissory target of therapeutic intervention. Actually, the PI3K inhibitors

From the Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

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**Reprints:** Patricio Aller, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040-Madrid, Spain; e-mail: [aller@cib.csic.es](mailto:aller@cib.csic.es).

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LY294002 and wortmannin were observed to exert antitumor activity in animal cell models<sup>15</sup>; and phase 1 and 2 clinical trials using rapamycin and rapamycin derivatives, which target the Akt downstream kinase mammalian target of rapamycin (mTOR), are ongoing.<sup>17</sup>

Of note, protein kinases and intracellular GSH are not unrelated factors. Thus, Sordet et al<sup>18</sup> reported that the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) potentiated the As<sub>2</sub>O<sub>3</sub> toxicity by decreasing GSH content in U-937 human promonocytic leukemia cells. These observations were corroborated by us, and we also demonstrated that the TPA-provoked GSH decrease was apparently mediated by ERK activation.<sup>19</sup> In addition, other authors demonstrated that PI3K inhibitors abrogated the insulin-provoked increase in GSH levels in rat cardiac myocytes<sup>20</sup> and hepatocytes.<sup>21</sup> These findings led us to examine whether GSH may represent a target of PI3K/Akt in myeloid cells, and if so, whether GSH mediates the changes in viability derived of PI3K/Akt disruption. The obtained results indicate that pharmacologic inhibitors of PI3K/Akt cause intracellular GSH depletion, increase peroxide accumulation, and potentiate the apoptotic action of As<sub>2</sub>O<sub>3</sub> in a GSH-dependent manner, in U-937 promonocytes and other myeloid leukemia cell lines.

## Materials and methods

### Chemicals

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Monochlorobimane, dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA), and rhodamine 123 (R123) were obtained from Molecular Probes (Eugene, OR). DAPI (4,6-diamino-2-phenylindole) was obtained from Serva (Heidelberg, Germany). The kinase inhibitors PD98059, U0126, SB203580, SP600125, LY294002, wortmannin, and 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt5); the caspase-3-specific substrate *N*-acetyl-Asp-Glu-Val-Asp-*P*-nitroanilide (Ac-DEVD-*p*NA); the caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-Fmk); and rabbit anti-human glutathione S-transferase P1-1 polyclonal antibody (pAb) were obtained from Calbiochem (Darmstadt, Germany). Rabbit polyclonal antibodies against human Akt, phospho-Akt (Ser<sup>473</sup>), p44/42 MAPK, and phospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-pigeon cytochrome *c* monoclonal antibody (mAb) clone 7H8.2C12, mouse anti-Bax mAb clone 6A7, and rabbit anti-rat Bcl-X pAb were obtained from BD PharMingen (San Diego, CA). Mouse anti-human Bcl-2 (100) mAb and rabbit anti-human Bax (N-20) pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human 70-kDa heat shock protein (HSP70) mAb (clone C92F3A-5, which specifically recognizes the stress-inducible form of HSP70) and mouse anti-human HSP27 mAb were obtained from StressGen Biotechnologies (Victoria, BC). All peroxidase- and fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) antibodies were obtained from DAKO Diagnostics (Barcelona, Spain). All other reagents were from Sigma (Madrid, Spain).

### Cells and treatments

The human leukemia cell lines U-937 (promonocytic),<sup>22</sup> HL-60 (myelomonocytic),<sup>23</sup> and NB4 (acute promyelocytic)<sup>24</sup>; and Bcl-2-transfected U-937 cells (U4 clone, kindly provided by Dr Jacqueline Bréard, Institut National de la Santé et de la Recherche Médicale [INSERM] 461, Chatenay Malabry, France)<sup>25</sup> were routinely grown in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated calf serum, 0.2% sodium bicarbonate, and antibiotics in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. For experiments, 16 to 24 hours before the initiation of the treatments the cell concentration was adjusted at approximately 10<sup>5</sup> cells/mL. Stock solutions of TPA (20 mM), camptothecin (10 mM), PD98059 (20 mM), U0126 (2.63 mM),

SB203580 (20 mM), SP600125 (20 mM), LY294002 (20 mM), wortmannin (1 mM), Akt5 (20 mM), Z-VAD-Fmk (25 mM), monochlorobimane (200 mM), Ac-DEVD-*p*NA (5 mM), *N*-acetyl-L-cysteine (NAC, 3 M), and lonidamine (100 mM) were prepared in dimethyl sulfoxide; a stock solution of cis-platinum(II)-diammine dichloride (cisplatin, 3.3 mM) was prepared in distilled water; and a stock solution of H<sub>2</sub>DCFDA (5 mM) was prepared in ethanol. All of these solutions were stored at -20°C. Stock solutions of DAPI (10 μg/mL), propidium iodide (PI, 1 mg/mL), and R123 (1 mg/mL) were prepared in phosphate-buffered saline (PBS); and a stock solution of As<sub>2</sub>O<sub>3</sub> (100 mM) was prepared in distilled water. These solutions were stored at 4°C. DL-buthionine-S, *R*-sulfoximine (BSO), and ascorbic acid (AA) were dissolved in distilled water at 50 and 10 mM, respectively, just before application.

### Flow cytometry

The analysis of samples was carried out using an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to FITC, H<sub>2</sub>DCFDA, and R123 were collected with a 525-nm band pass filter and the signal corresponding to PI, with a 620-nm band pass filter.

### Determination of apoptosis

Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G<sub>1</sub> DNA content. To examine chromatin structure, cells were fixed with ethanol, stained with DAPI, and examined by fluorescence microscopy. To measure DNA content, cells were permeabilized, stained with PI, and examined by flow cytometry. These procedures were described in detail elsewhere.<sup>26</sup>

### Measurement of caspase-3 activity

Samples of 4 × 10<sup>6</sup> cells were collected by centrifugation, washed twice with ice-cold PBS, resuspended in 50 μL ice-cold lysis buffer (1 mM dithiothreitol, 0.03% nonidet P-40 [vol/vol] in 50 mM Tris [tris(hydroxymethyl)aminomethane, pH 7.5]), kept on ice for 30 minutes, and finally centrifuged at 14000g for 15 minutes at 4°C. Samples containing aliquots of the supernatants (corresponding to 10 μg total protein), 8 μL caspase substrate (Ac-DEVD-*p*NA), and PBS to complete 200 μL were prepared in triplicate in 96-well microtiter plates and incubated for one hour at 37°C. The absorption was measured by spectrometry at 405 nm.

### Determination of active Bax

Cells were fixed with 0.35% (vol/vol) formaldehyde and permeabilized with 0.1% (wt/vol) saponin in PBS for 5 minutes on ice. After incubation for 30 minutes at 4°C with anti-Bax antibody clone 6A7, and for 30 minutes at 4°C with FITC-conjugated anti-IgG antibody, the fluorescence was estimated by flow cytometry. The 6A7 clone recognizes a NH<sub>2</sub>-terminal region of Bax that is occluded under normal conditions, but that is exposed as a consequence of changes in conformation associated to Bax translocation to mitochondria in stressed cells.<sup>27</sup>

### Measurement of peroxide accumulation and mitochondrial transmembrane potential

Intracellular oxidation was measured using the fluorescent probe H<sub>2</sub>DCFDA, which is preferentially sensitive to peroxides.<sup>28</sup> With this aim, the cells were collected by centrifugation and incubated for one hour at 37°C in red phenol-lacking RPMI medium containing 5 μM H<sub>2</sub>DCFDA, centrifuged again, and resuspended in PBS. The fluorescence was then measured by flow cytometry.

To measure the mitochondrial transmembrane potential (ΔΨ<sub>m</sub>), the cells were incubated for 20 minutes at 37°C with PBS containing 1 μg/mL R123. After washing with PBS, the cells were resuspended in PBS and the fluorescence was measured by flow cytometry. Under these conditions, incubation with the depolarizing agent carbonyl cyanide *P*-(trifluoromethoxy) phenylhydrazone (100 μM) greatly decreased ΔΨ<sub>m</sub>.

### Measurement of glutathione content and glutathione S-transferase $\pi$ activity

The intracellular reduced glutathione (GSH) content was currently determined by fluorometry after cell loading with monochlorobimane, following the previously described procedure.<sup>29</sup> In some experiments, the content of both reduced glutathione (GSH) and glutathione disulfide (GSSG) was determined by high-pressure liquid chromatography (HPLC), according to the procedure described by Fariss and Reed,<sup>30</sup> using a 5- $\mu$ M Spherisorb  $NH_2$  analytical column (Water, Milford, MA).

The glutathione S-transferase (GST)  $\pi$  activity was measured in cell lysates using 1-chloro-2,4-dinitrobenzene and GSH as substrates,<sup>31</sup> according to the description of Jing et al.<sup>5</sup>

### Immunoblot assays

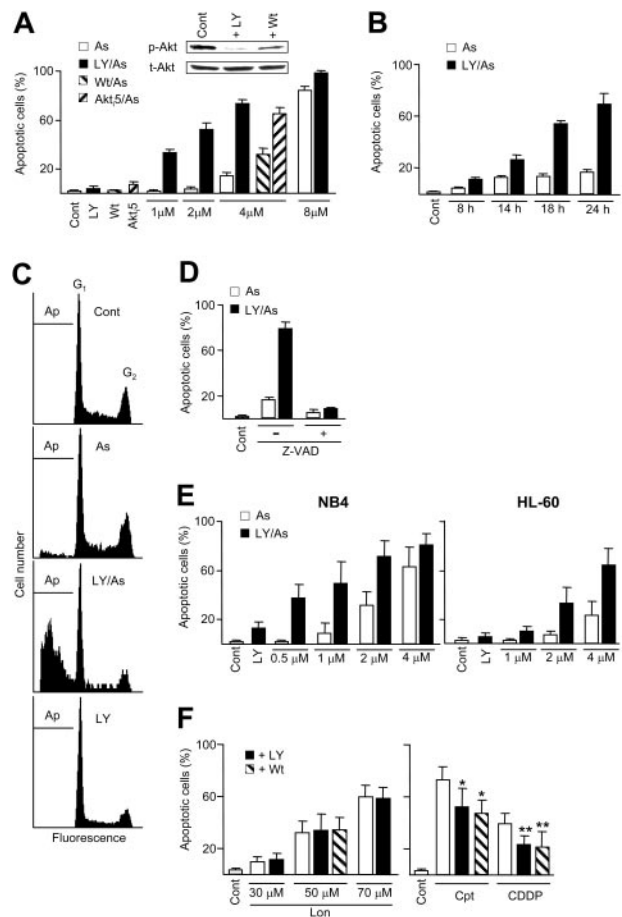
To obtain total cellular protein extracts, cells were collected by centrifugation, washed with PBS, and lysed by 5-minute heating at 100°C followed by sonication in Laemmli buffer containing a protease inhibitor cocktail, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. To obtain cytosolic extracts (aimed at determining cytochrome *c* release from mitochondria), cells were collected for centrifugation; resuspended in 100  $\mu$ L ice-cold PBS containing 80 mM KCl, 250 mM sucrose, and 200  $\mu$ g/mL digitonin; and kept on ice for 5 minutes. After centrifugation (10 000g for 15 minutes at 4°C) the pellet was discarded. Fractions of the total or cytosolic extracts, containing equal protein amounts, were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected, as previously described.<sup>32</sup>

## Results

### Apoptosis induction by $As_2O_3$ and other antitumor drugs, and its modulation by PI3K/Akt inhibitors

First we analyzed the capacity of  $As_2O_3$ , alone or in combination with the PI3K inhibitors LY294002 and wortmannin, to induce apoptosis in U-937 cells. LY294002 and wortmannin, which are known to inhibit PI3K activity with different action mechanisms,<sup>15</sup> were used at 30 and 0.5  $\mu$ M, respectively. These concentrations were adopted on the grounds of earlier publications, and their efficacy was here corroborated by measuring the level of phosphorylated Akt (Figure 1A, inset). It was observed that  $As_2O_3$  caused a concentration-dependent (Figure 1A) and time-dependent (Figure 1B) increase in the frequency of cells with fragmented chromatin, which is characteristic of apoptosis. Some accumulation of cells at the  $G_2$  phase of the growth cycle was also detected at treatments longer than 24 hours (result not shown). Treatments for 24 hours with the PI3K inhibitors alone were innocuous (Figure 1A). Longer treatments (48-72 hours) did not cause cell death, but decreased cell proliferation by accumulating cells at the  $G_1$  phase of the growth cycle (result not shown). When used in combination, the PI3K inhibitors greatly potentiated the generation of apoptosis by  $As_2O_3$ , with maximum efficacy in the case of LY294002 (Figure 1A-B). These conclusions were confirmed by measuring the frequency of cells with sub- $G_1$  DNA content (Figure 1C, and results not shown), which is also an indicator of apoptosis. Moreover, the pan-caspase inhibitor Z-VAD-Fmk abrogated the toxicity of  $As_2O_3$  alone or with LY294002 (Figure 1D), corroborating that the measured cell death is a bona fide caspase-dependent, typical apoptosis. The potentiation of  $As_2O_3$ -provoked apoptosis was also observed using 30  $\mu$ M Akt5, a novel inhibitor that is known to directly prevent Akt phosphorylation<sup>33</sup> (Figure 1A).

To determine whether the potentiation of  $As_2O_3$  toxicity by PI3K/Akt inhibitors was a cell line-specific response, experiments were carried out using human leukemia HL-60 (myelomonocytic)



**Figure 1. Generation of apoptosis by antitumor drugs and PI3K/Akt inhibitors in human myeloid leukemia cell lines.** (A) Frequency of apoptotic cells, as determined by chromatin fragmentation, in untreated U-937 cell cultures (Cont); in cultures treated for 24 hours with LY294002 (LY), wortmannin (Wt), or Akt5 alone; and in cultures treated for 24 hours with the indicated concentrations of  $As_2O_3$ , either alone (As) or in combination with LY294002 (LY/As), wortmannin (Wt/As), or Akt5 (Akt5/As). The inset represents the relative level of total and phosphorylated Akt (t-Akt and p-Akt, respectively) in total cellular extracts obtained from untreated cells (Cont) and cells treated for 24 hours with LY294002 and wortmannin, as determined by immunoblot. (B) Frequency of apoptosis in U-937 cell cultures treated for the indicated time periods with 4  $\mu$ M  $As_2O_3$ , alone or in combination with LY294002. (C) Cell distribution according to their DNA content in untreated U-937 cell cultures (Cont) and in cultures treated for 24 hours with 4  $\mu$ M  $As_2O_3$  and LY294002, alone and in combination. The fraction of cells with sub- $G_1$  DNA content (Ap) is considered as apoptotic. (D) Frequency of apoptosis in U-937 cell cultures treated for 24 hours with 4  $\mu$ M  $As_2O_3$ , alone or with LY294002, either in the absence (-) or the presence (+) of 50  $\mu$ M Z-VAD-FMK (Z-VAD). (E) Frequency of apoptosis in human NB4 and HL-60 cell cultures, treated for 24 hours with LY294002 and the indicated concentrations of  $As_2O_3$ , alone or in combination. (F) Frequency of apoptosis in U-937 cell cultures treated for 24 hours with the indicated concentrations of lonidamine (Lon), 20 nM camptothecin (Cpt), and 4  $\mu$ M cisplatin (CDDP), alone (-) and in combination with LY294002 or wortmannin. The asterisks indicate significant differences (\* $P < .02$ , \*\* $P < .0005$ , Student *t* test), in relation to cells treated with the correspondent antitumor drug alone. LY294002, wortmannin, and Akt5 were used at 30, 0.5, and 30  $\mu$ M, respectively. All agents (antitumor drugs, kinase inhibitors, and Z-VAD-FMK) were applied simultaneously. The data in panels A-B and D-F represent the mean  $\pm$  SD of at least 3 determinations. The data in panel C and the inset in panel A are representative of 2 determinations with similar results.

and NB4 (acute promyelocytic) cells. It was observed that LY294002 also potentiated the apoptotic action of  $As_2O_3$  in both cell lines (Figure 1E). NB4 cells exhibited a higher sensitivity to  $As_2O_3$  than U-937 and HL-60 cells, as earlier reported.<sup>4,5</sup>

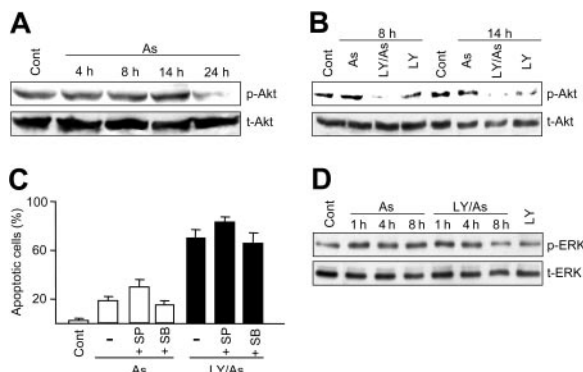
To determine whether the potentiation of  $As_2O_3$  toxicity was a drug-specific response, experiments were carried out using lonidamine—an agent that, as  $As_2O_3$ , directly targets the mitochondria<sup>34</sup>—and the DNA-specific antitumor drugs camptothecin and

cisplatin. For homogeneity, we adopted a similar experimental design as in the case of  $As_2O_3$ , namely prolonged treatments (24 hours) with relatively low drug concentrations. It was found that, by contrast to  $As_2O_3$ , the PI3K inhibitors did not modify the apoptotic action of lonidamine, and they even decreased the apoptotic action of camptothecin and cisplatin, as measured by chromatin fragmentation (Figure 1F). This conclusion was corroborated by measuring the frequency of cells with decreased (sub- $G_1$ ) DNA content by flow cytometry assays, which also indicated that camptothecin and cisplatin provoked  $G_2$  arrest, while lonidamine did not cause significant phase-specific blockade (results not shown).

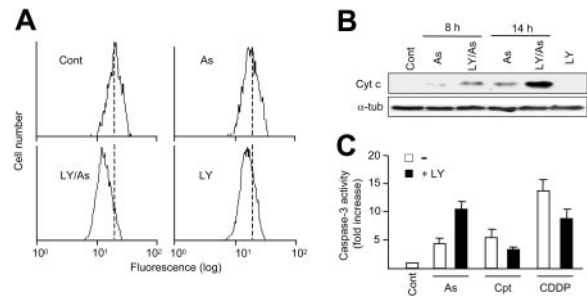
### Protein kinase activation

It was reported that cytotoxic agents may affect Akt phosphorylation/activation in U-937 cells.<sup>35,36</sup> For this reason, we wanted to measure Akt phosphorylation upon treatment with  $As_2O_3$ , in the absence and presence of LY294002. Treatment with  $As_2O_3$  alone caused a late decrease (24 hours) in Akt phosphorylation (Figure 2A). On the other hand, treatment with  $As_2O_3$  plus LY294002 caused an earlier decrease in phosphorylation (8 hours and thereafter), with higher efficacy than treatment with LY294002 alone (Figure 2B).

Earlier publications indicated that PI3K/Akt may negatively regulate MAPK pathways.<sup>37-39</sup> Hence, we queried whether the potentiation of  $As_2O_3$  toxicity by PI3K/Akt inhibitors could be mediated by MAPK activation. To analyze this possibility, experiments were carried out using appropriate MAPK inhibitors, namely 10  $\mu$ M PD98059 and 2.5  $\mu$ M U0126 (specific for mitogen-induced extracellular kinase [MEK]/ERK), 10  $\mu$ M SB20358 (specific for p38), and 10  $\mu$ M SP600125 (specific for Jun N-terminal kinase [JNK]). These concentrations proved to efficaciously block kinase activation in a preceding work.<sup>19</sup> It was found that SB20358 and SP20358, which were nontoxic when used alone or in combination with LY294002 (results not shown), did not affect or only minimally modified the generation of apoptosis by  $As_2O_3$  plus LY294002 (Figure 2C), indicating that the potentiation of  $As_2O_3$  toxicity may not be explained by a possible p38 or JNK activation. Experiments with MEK/ERK inhibitors could not be carried out



**Figure 2. Akt and ERK activation, and effect of MAPK inhibitors.** (A-B) Relative level of total and phosphorylated Akt in extracts (40  $\mu$ g protein per lane) obtained from untreated U-937 cells (Cont) and cells treated for the indicated time periods with 4  $\mu$ M  $As_2O_3$  (A); and from cells treated for the indicated time periods with  $As_2O_3$  and LY294002, alone and in combination (B). (C) Frequency of apoptotic cells (mean  $\pm$  standard deviation of 4 determinations) in cultures treated for 24 hours with 4  $\mu$ M  $As_2O_3$ , alone or with LY294002, either in the absence (-) or the presence (+) of 10  $\mu$ M SP600125 (SP) or 10  $\mu$ M SB203580 (SB). (D) Relative level of total (t-ERK) and phosphorylated (p-ERK) ERKs in extracts (10  $\mu$ g protein per lane) obtained from untreated cells and cells treated for the indicated time periods with  $As_2O_3$  and LY294002, alone and in combination. All drugs were applied simultaneously. All other conditions were as in Figure 1.



**Figure 3. Disruption of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ), cytochrome *c* release, and caspase-3 activation.** (A) The figure shows the changes in  $\Delta\Psi_m$  in U-937 cells treated for 14 hours with 4  $\mu$ M  $As_2O_3$  and LY294002, alone or in combination, as determined by flow cytometry after cell loading with R123. The vertical, dotted lines represent the mean fluorescence value in untreated cells (Cont), to better discern the displacement caused by the treatments. (B) The blot shows the presence of cytochrome *c* (Cyt *c*) in cytosolic extracts (25  $\mu$ g protein per lane) obtained from untreated cells (Cont), from cells treated for 14 hours with LY291002 alone, and from cells treated for the indicated time periods with 4  $\mu$ M  $As_2O_3$ , alone or in combination with LY294002. The level of  $\alpha$ -tubulin ( $\alpha$ -tub) was also measured as a control. (C) The figure shows the relative caspase-3 activity, as determined using Ac-DEVD-pNA as substrate, in extracts from untreated cells (Cont) and cells treated for 24 hours with 4  $\mu$ M  $As_2O_3$ , 20 nM camptothecin, and 4  $\mu$ M cisplatin, alone (-) or in combination with LY294002 (+LY). The results (mean  $\pm$  standard deviation of 3 determinations) are represented in relation to the control, which was given the arbitrary value of one. All other conditions were as in Figure 1.

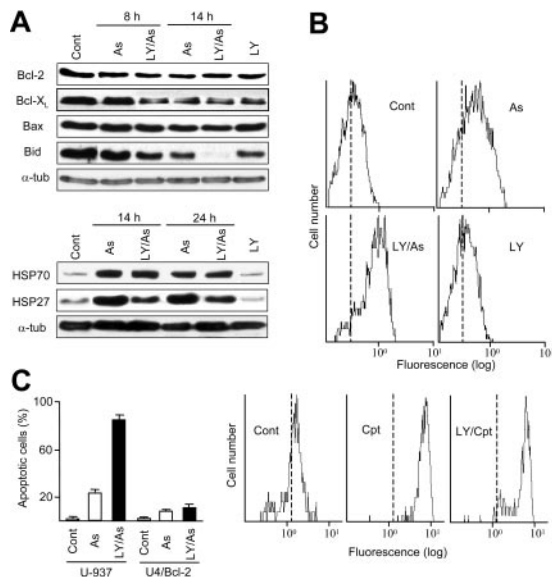
since PD98059 and U0126, although nontoxic when used alone, were extremely toxic in combination with LY294002, even in the absence of  $As_2O_3$  (result not shown). Nonetheless, immunoblot assays revealed that ERK phosphorylation was not affected by LY294002, either alone or in combination with  $As_2O_3$  (Figure 2D), a result that apparently excludes a possible regulation via the MEK/ERK pathway.

### Modulation of mitochondria-related events

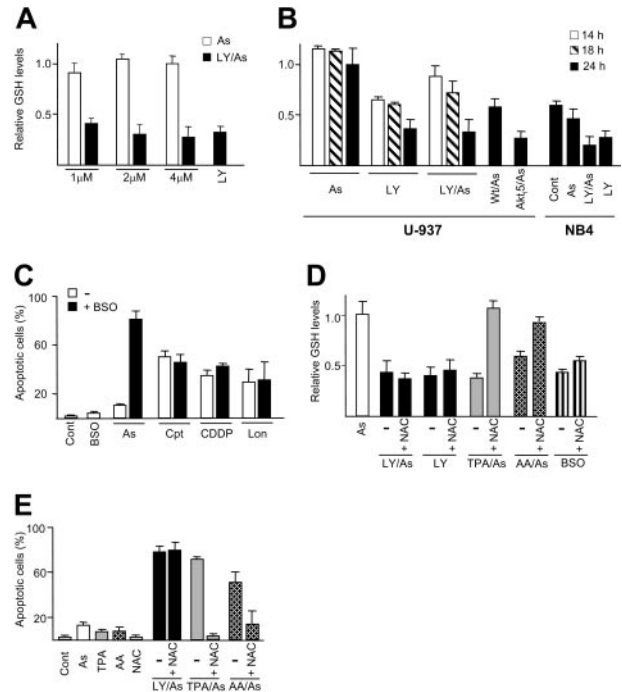
It is known that  $As_2O_3$  directly targets the mitochondria by interacting with the permeability transition pore.<sup>40</sup> This led us to examine 2 critical mitochondria-associated regulatory events, namely the dissipation of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) and the release of cytochrome *c*, which initiates apoptosis execution along the “intrinsic” pathway.<sup>41</sup> Figure 3A shows the alteration in  $\Delta\Psi_m$ , as measured by flow cytometry using the cationic dye R123. Treatment for 14 hours with  $As_2O_3$  alone caused a slight  $\Delta\Psi_m$  decrease, which was potentiated by cotreatment with LY294002. Of note, treatment with the inhibitor alone, although nontoxic (Figure 1), sufficed to slightly reduce  $\Delta\Psi_m$ . Qualitatively similar results were obtained using the cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) instead of R123 (results not shown). Figure 3B shows the release of cytochrome *c* from mitochondria to the cytosol, as revealed by immunoblot using cytosolic extracts.  $As_2O_3$  slightly induced cytochrome *c* release, which was greatly potentiated by cotreatment with LY294002. By contrast to  $\Delta\Psi_m$  dissipation, no cytochrome *c* release could be detected in cells treated with LY294002 alone. The changes in cytochrome *c* location were followed by the activation of the executioner caspase-3, as determined by measuring DEVDase activity in cellular extracts. Thus, extracts from cells treated with  $As_2O_3$  alone exhibited a moderate increase in DEVDase activity, which was higher in cells treated with  $As_2O_3$  plus LY294002 (Figure 3C). In contrast, the PI3K inhibitor did not potentiate, and instead decreased, DEVDase activity in combination with camptothecin or cisplatin, which agrees with the changes in apoptosis (indicated in Figure 1).

The release of cytochrome *c* is regulated by proteins of the Bcl-2 family, which may either inhibit (eg, the antiapoptotic

proteins Bcl-2 and Bcl- $X_L$ ) or promote (eg, the proapoptotic proteins Bax and Bid) the process.<sup>41</sup> Bax remains inactive in the cytosol, and to be functional requires changes in conformation and translocation to the mitochondrial membrane.<sup>27</sup> Bid is activated by cleavage followed by translocation to the mitochondrial membrane.<sup>42</sup> In addition, the “heat-shock” proteins HSP27 and HSP70 also inhibit apoptosis by blocking cytochrome *c* and/or other elements of the intrinsic pathway.<sup>43</sup> Hence, experiments were carried out to measure the expression and modification of these proteins. The results, represented in Figure 4, were as follows: (1) Treatment with  $As_2O_3$ , with or without LY294002, did not significantly modify the total Bcl-2 and Bax levels, but caused a slight decrease in Bcl- $X_L$  (Figure 4A). (2)  $As_2O_3$  caused Bax activation, and this activation was further enhanced by cotreatment with LY294002, as determined by flow cytometry using the 6A7 antibody. By contrast, the PI3K inhibitor did not potentiate the camptothecin-provoked Bax activation (Figure 4B). (3) LY294002 also potentiated the  $As_2O_3$ -provoked Bid cleavage, using as criterion the loss of the 21-kDa proform<sup>26</sup> (Figure 4A). (4)  $As_2O_3$  increased HSP70 and HSP27 expression. However, while HSP70 was not affected by LY294002, the PI3K inhibitor reduced the basal HSP27 level as well as the increase caused by  $As_2O_3$  (Figure 4A). Hence, Bax and Bid activation, and HSP27 decrease, may contribute to the increased cytochrome *c* release and apoptosis execution in  $As_2O_3$  plus LY294002-treated cells. The possible contribution of Bad, another proapoptotic member of the Bcl-2 family (the activity of which is regulated by Akt-mediated changes in phosphorylation),<sup>16</sup> could not be determined since phosphorylated Bad was below detection level in our immunoblot assays.



**Figure 4. Expression and modification of Bcl-2 and HSP family members, and effect of Bcl-2 overexpression.** (A) Relative levels of Bcl-2, Bcl- $X_L$ , Bax, Bid (21-kDa proform), HSP70, HSP27, and  $\alpha$ -tubulin (used as a control) in total cellular extracts (15  $\mu$ g per lane) obtained from untreated U-937 cells (Cont), from cells treated for 14 hours with LY294002 alone, and from cells treated for the indicated time periods with 4  $\mu$ M  $As_2O_3$ , alone or in combination with LY294002, as determined by immunoblot. (B) Relative level of activated Bax in untreated cells (Cont), in cells treated for 14 hours with LY294002 alone, and in cells treated for 14 hours with  $As_2O_3$  and camptothecin, alone or in the presence of LY294002, as determined by flow cytometry using the 6A7 antibody. The vertical, dotted lines represent the mean fluorescence value in the control, to better discern the displacement caused by the treatments. (C) Frequency of apoptotic cells (mean  $\pm$  standard deviation of 3 determinations) in nontransfected (U-937) and Bcl-2-transfected (U4/Bcl-2) U-937 cell cultures at 24 hours of treatment with 4  $\mu$ M  $As_2O_3$ , alone or in combination with LY294002. All other conditions were as in Figure 1.



**Figure 5. Modulation of GSH level and its relationship with apoptosis.** (A-B) Relative GSH levels, as determined by monochlorobimane derivatization, in U-937 cell cultures treated for 24 hours with LY294002 and the indicated concentrations of  $As_2O_3$ , alone and in combination (A); and in U-937 and NB4 cell cultures treated for the indicated time periods with 4  $\mu$ M (U-937) and 2  $\mu$ M (NB4)  $As_2O_3$  alone, with LY294002 alone, and with  $As_2O_3$  plus either LY294002, wortmannin, or Akt5 (B). All results are expressed in relation to untreated U-937 cell cultures (approximate GSH content, 9 nmol/ $10^6$  cells), which received the arbitrary value of one. (C) Frequency of apoptotic cells in U-937 cell cultures treated for 24 hours with BSO alone, and with  $As_2O_3$ , camptothecin, cisplatin, and lonidamine, either alone (–) or in combination with BSO. (D) Relative GSH levels in U-937 cells treated for 24 hours with LY294002, with BSO, and with the combination of  $As_2O_3$  plus either LY294002, TPA, or ascorbic acid (AA), in the absence (–) or the presence of NAC (+NAC). (E) Frequency of apoptotic cells in U-937 cell cultures treated for 24 hours with  $As_2O_3$  alone, TPA alone, AA alone, and NAC alone, and with the combinations of  $As_2O_3$  plus either LY294002, TPA, or AA, in the absence (–) or the presence of NAC. BSO, TPA, AA, and NAC were used at 1 mM, 20 nM, 150  $\mu$ M, and 10 mM, respectively. All drugs were applied simultaneously. The data represent the means  $\pm$  standard deviation of at least 3 determinations. All other conditions were as in Figure 1.

Finally, it was observed that the frequency of apoptosis was greatly reduced in Bcl-2-transfected U-937 cells (which, according to our control observations, possess an 8-fold increase in Bcl-2 content in relation to the nontransfected cells) (Figure 4C). This indicates that the generation of apoptosis by  $As_2O_3$  and its potentiation by LY294002 is Bcl-2 regulated, as expected in the intrinsic pathway.

### Changes in glutathione content

As mentioned in the “Introduction,” the toxicity of  $As_2O_3$  is greatly dependent on the intracellular GSH content, which in turn may be affected by changes in protein kinase activities. For these reasons, determinations using the GSH-sensitive fluorescent probe monochlorobimane were carried out to analyze the possible effects of  $As_2O_3$  and PI3K/Akt inhibitors on intracellular GSH in U-937 cells. As indicated in Figure 5A-B, the GSH content was not reduced by treatment with 1 to 4  $\mu$ M  $As_2O_3$  alone, but was considerably decreased by treatment with LY294002, either alone or in combination with  $As_2O_3$ . This response was corroborated using combinations of  $As_2O_3$  plus wortmannin or Akt5, and using NB4 and HL-60 cells instead of U-937 cells (Figure 5B, and results not shown). The importance of GSH depletion as mediator of apoptosis

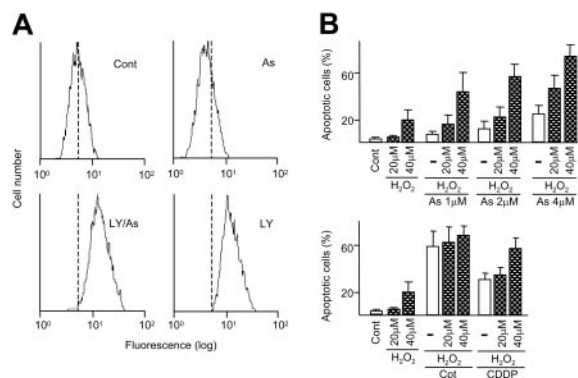
induction in our experimental conditions was confirmed using BSO, a specific inhibitor of  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) activity, the rate-limiting enzyme of GSH biosynthesis. Preliminary determinations indicated that a 24-hour treatment with 1 mM BSO caused approximately a 60% inhibition in the intracellular GSH content (result not shown). It was observed that treatment with BSO alone was nontoxic, but as expected cotreatment with BSO increased the apoptotic action of  $As_2O_3$ , in the same manner as the PI3K/Akt inhibitors. By contrast, BSO failed to increase the apoptotic action of cisplatin, camptothecin, and lonidamine (Figure 5C). This result indicates that under the conditions used here, cisplatin, camptothecin, and lonidamine behave as GSH-insensitive drugs, and may therefore explain the inability of the PI3K inhibitors to potentiate their toxicity (Figure 1F).

To shed some light on the mechanisms by which PI3K/Akt inhibition could deplete GSH, determinations were carried out using NAC, an agent earlier used as a cysteine donor for GSH biosynthesis in experiments with  $As_2O_3$ .<sup>4,7</sup> In addition to LY294002 and BSO, in these assays we used TPA and AA, which were also reported to reduce GSH in myeloid cells and other cell systems.<sup>4,7,18,19</sup> The results are indicated in Figure 5D. NAC was unable to restore the GSH content in cells treated with BSO (which, as indicated above, blocks enzyme activity); and it was similarly ineffective in cells treated with LY294002, either alone or with  $As_2O_3$ . However, NAC restored the GSH content in TPA plus  $As_2O_3$ - and AA plus  $As_2O_3$ -treated cells. This suggests that, in contrast to TPA and AA, and in the same manner as BSO, PI3K/Akt inhibitors modulate GSH biosynthesis at levels other than substrate availability. Moreover, in good parallelism with these results, NAC abrogated the increase in toxicity in the case of TPA and AA, but not in the case of LY294009 (Figure 5E), confirming again the importance of GSH as a mediator of the increased apoptosis in cells treated with  $As_2O_3$  plus PI3K inhibitors.

Final information was obtained by means of HPLC. As indicated in Table 1, this technique allowed us to corroborate the capacity of LY294002 to cause GSH depletion, and also proved that the GSH decrease provoked by the PI3K inhibitor was not adequately compensated by an increase in GSSG. Nonetheless, the ratio of GSH to GSSG was diminished, especially in  $As_2O_3$  plus LY294002-treated cells, indicating that the treatments caused GSH oxidation to some degree.

### ROS accumulation and GST $\pi$ expression and activity

One of the roles of GSH is the scavenging of ROS.<sup>8</sup> Hence, we queried whether the GSH depletion derived from PI3K/Akt inhibition could result in ROS overaccumulation, which might in turn explain the increase in  $As_2O_3$  toxicity. To answer this question, flow cytometry determinations were carried out using the peroxide-sensitive fluorescent probe  $H_2DCFDA$ . The results are represented in Figure 6A: (1) treatment with  $As_2O_3$  alone, which as indicated in



**Figure 6. Peroxide accumulation and effects of hydrogen peroxide.** (A) Intracellular peroxide accumulation in untreated U-937 cells (Cont), in cells treated for 14 hours with LY294002 alone, and in cells treated for 14 hours with 4  $\mu M$   $As_2O_3$ , alone or in combination with LY294002, as determined by flow cytometry after cell loading with  $H_2DCFDA$ . The vertical, dotted lines represent the mean fluorescence value in the control, to better discern the displacement caused by the treatments. (B) Frequency of apoptotic cells (mean  $\pm$  standard deviation of at least 4 determinations) in U-937 cell cultures treated for 24 hours with 20 and 40  $\mu M$   $H_2O_2$  alone, and with 1 to 4  $\mu M$   $As_2O_3$ , camptothecin, and cisplatin, either alone (–) or in combination with  $H_2O_2$ . All other conditions were as in Figure 1.

Figure 5 did not decrease GSH, did not affect peroxide accumulation; (2) treatment with LY294002 alone, which as indicated in Figures 5 and 1 sufficed to decrease GSH but was not toxic per se, increased the intracellular peroxide accumulation; and (3) a similar increase was obtained by treatment with  $As_2O_3$  plus LY294002, which as indicated in Figures 5 and 1 decreased GSH and was highly toxic.

The preceding observations could indicate that while the cells tolerate a limited ROS overaccumulation without concomitant toxicity, such overaccumulation may facilitate the generation of apoptosis by  $As_2O_3$ . To investigate this possibility, experiments were carried out in which cells were cotreated with  $As_2O_3$  plus low  $H_2O_2$  concentrations (20–40  $\mu M$ ), which were per se innocuous or slightly toxic. In agreement with our hypothesis,  $H_2O_2$  potentiated or exerted a synergic effect with  $As_2O_3$ , at the concentrations of 2 to 4  $\mu M$ . By contrast,  $H_2O_2$  did not modify or exhibited only additive effects in combination with camptothecin and cisplatin (Figure 6B).

It has been indicated that GST $\pi$  is key element in the  $As_2O_3$  detoxification machinery.<sup>11,12</sup> For this reason, we asked whether the potentiation of  $As_2O_3$  toxicity by PI3K/Akt inhibitors involved an alteration in GST $\pi$  expression and/or activity. The results in Figure 7 indicate that both the enzyme content (as measured by immunoblot assays) and activity remained unaltered or were slightly increased upon treatment with  $As_2O_3$  and LY294002, alone and in combination, when compared with the values in untreated cells.

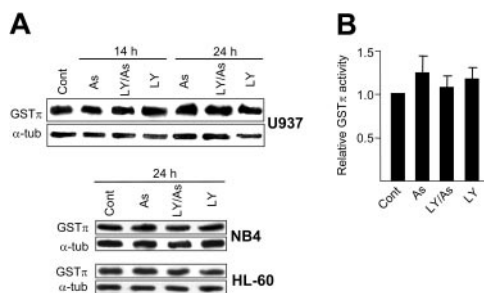
## Discussion

The results in this work indicate that the generation of apoptosis by low concentrations of  $As_2O_3$  is potentiated by cotreatment with PI3K/Akt inhibitors (LY294002, wortmannin, Akt5) in U-937 promonocytic and other myeloid leukemia cells. In spite of the importance of PI3K/Akt as a survival-promoting pathway, treatment with the inhibitors alone did not significantly cause cell death, in contrast with the results sometimes obtained in other cell models.<sup>44,45</sup> A possible explanation is that the intact MEK/ERK pathway provides enough survival signals to keep the cell viability. In fact, the simultaneous inactivation of PI3K/Akt and MEK/ERKs by cotreatment with LY294002 plus either PD98059 or U0126

**Table 1. Effect of treatment with LY-294002 alone (LY) and LY294002 plus  $As_2O_3$  (LY/As) on the intracellular GSH and GSSG content in U-937 cells**

	Control	LY	LY/As
No.	6	6	3
GSH, nmol/mg protein	75.1 $\pm$ 11.7	45.7 $\pm$ 6.1	37.1 $\pm$ 6.6
GSSG, nmol/mg protein	1.5 $\pm$ 0.4	1.6 $\pm$ 0.6	3.1 $\pm$ 0.8
GSH/GSSG, nmol/mg protein	50.5	28.6	12.0

The values (nmol/mg protein) were obtained at 24 hours of treatment. For other conditions, see Figure 1 legend.



**Figure 7. GST $\pi$  expression and activity.** (A) Relative GST $\pi$  levels in extracts (10  $\mu$ g protein per lane) obtained from untreated cells (Cont) and cells treated for the indicated time periods with LY294002 and As<sub>2</sub>O<sub>3</sub>, alone and in combination. As<sub>2</sub>O<sub>3</sub> was used at 4  $\mu$ M in the case of U-937 and HL-60, and at 2  $\mu$ M in the case of NB4 cell cultures. (B) GST $\pi$  activity in untreated U-937 cells and cells subjected for 24 hours to the same treatments as in panel A. The results (mean  $\pm$  standard deviation of 3 determinations) are expressed in relation to untreated cells (main value, 44.5  $\pm$  6.9 nmol/mg protein/min), which received the arbitrary value of one. All other conditions were as in Figure 1.

drastically induced apoptosis in U-937 cells. The potentiation by PI3K/Akt inhibitors of the As<sub>2</sub>O<sub>3</sub>-provoked apoptosis represented a drug-specific response, which could not be reproduced using lonidamine, camptothecin, and cisplatin. This result was unexpected, since with few exceptions PI3K/Akt inhibition was earlier reported to potentiate the lethality of cytotoxic drugs in myeloid cells.<sup>35,46,47</sup> Such discrepancy might be explained by differences in the experimental designs used. While other works used elevated drug concentrations for short time periods (typically 4-6 hours),<sup>35,46,47</sup> in the present study we used relatively low concentrations during a longer time period (24 hours). Under these conditions, the alterations of cell cycle regulatory events derived of PI3K/Akt inhibition may considerably affect the action of the antitumor drugs, which often behave as cycle phase-specific drugs (eg, G<sub>2</sub>-arrest by As<sub>2</sub>O<sub>3</sub>, camptothecin, and cisplatin in U-937 cells, which are p53-null cells<sup>48</sup>). Actually, this argument was earlier used to explain the LY294002-provoked attenuation of toxicity upon long-term cisplatin treatment in 32D murine myeloid cells.<sup>49</sup> However, while such explanation might account for the discrepancies between our and other studies, it does not satisfactorily explain the different modulation by PI3K inhibitors of apoptosis induction by As<sub>2</sub>O<sub>3</sub> and the other assayed drugs, observed in the present work. In accordance with its property as a mitochondria-targeting drug, the action of As<sub>2</sub>O<sub>3</sub> and the potentiation by LY294002 exhibited typical characteristics of the “intrinsic” pathway—namely increased cytochrome *c* release, negative regulation by Bcl-2, and positive regulation by Bax. Moreover, while the mechanism by which LY294002 inhibits HSP27 expression is still unknown, such inhibition may also contribute to the increase in toxicity, since HSP27 is an antiapoptotic protein.<sup>43</sup> Nonetheless, the possibility that the “extrinsic” (receptor-mediated) pathway could also participate in the process may not be excluded, since As<sub>2</sub>O<sub>3</sub> was reported to activate this pathway in some cell types.<sup>50,51</sup> This possibility is presently under investigation in our laboratory.

In addition, the present results demonstrate that the PI3K/Akt signaling pathway is required to maintain the normal GSH

metabolism in myeloid cells, since the basal GSH content was reduced upon treatment with PI3K/Akt inhibitors, either alone or in combination with As<sub>2</sub>O<sub>3</sub>. Although our mechanistic studies on GSH regulation by PI3K/Akt are still preliminary, it may be concluded that (1) GSH depletion was not a mere consequence of cell death, since a 24-hour treatment with LY294002 alone, which was nontoxic, sufficed to considerably decrease GSH; (2) the GSH depletion was not adequately compensated by the appearance of the oxidized form, GSSG; and (3) GSH was not regulated at the level of substrate availability since (in contrast to TPA and AA, and in the same manner as BSO) the LY294002-provoked GSH depletion was not prevented by NAC. Therefore, a possible explanation is that PI3K/Akt may directly regulate  $\gamma$ -GCS, either at the level of enzyme synthesis or activity. Actually, PI3K inhibitors were recently reported to abrogate the insulin-mediated increase in  $\gamma$ -GCS catalytic subunit synthesis in rat hepatocytes,<sup>21</sup> and  $\gamma$ -GCS activity is susceptible to modulation by changes in phosphorylation.<sup>52</sup> Whatever the mechanism, GSH depletion appears to be an important factor in explaining the selective potentiation by PI3K/Akt inhibitors of apoptosis induction by As<sub>2</sub>O<sub>3</sub>, but not by other antitumor drugs. In fact, in contrast to As<sub>2</sub>O<sub>3</sub>, under the hereby assayed conditions, camptothecin, cisplatin, and lonidamine behaved as GSH-insensitive drugs, as indicated by the inability of the GSH-specific agent BSO to increase their toxicity. Due to the multiplicity of functions of glutathione, it is conceivable that different mechanisms may participate in potentiating As<sub>2</sub>O<sub>3</sub> toxicity under GSH-depleting conditions. Thus, in our experiments the LY294002-provoked GSH decrease was paralleled by an increase in intracellular peroxides. This is important since ROS elevation has been reported to sensitize leukemic cells to As<sub>2</sub>O<sub>3</sub>-provoked apoptosis<sup>53</sup>—a response here corroborated by us by cotreatment with low H<sub>2</sub>O<sub>2</sub> concentrations. In addition, since GSH directly reacts with As<sub>2</sub>O<sub>3</sub>,<sup>10</sup> GSH depletion may result in an increase in free, acting intracellular As<sub>2</sub>O<sub>3</sub> concentration and hence in toxicity, even in the absence of significant alterations in GST $\pi$  expression and activity. Of course, the GSH-based regulation does not exclude the concurrence of additional mechanisms (eg, alterations of critical cell-cycle regulatory events), which as indicated earlier in this section might explain the unusual attenuation by PI3K inhibitors of camptothecin and cisplatin toxicity, observed in this and other reports.<sup>49</sup>

In summary, the present study provides the first demonstration that GSH is a target of the PI3K/Akt in myeloid leukemia cells, and as such it may partially explain the capacity of PI3K/Akt inhibitors to selectively potentiate the apoptotic action of the antileukemic drug As<sub>2</sub>O<sub>3</sub>. We believe that this property might provide a rationale to improve the efficacy of therapies based on the use of pharmacologic inhibitors of the PI3K/Akt pathway.

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