Arsenic trioxide (As$_2$O$_3$) caused apoptosis in U-937 human promonocytic cells. This effect was potentiated by the simultaneous addition of the glutathione (GSH) synthesis inhibitor DL-buthionine-(R,S)-sulfoximine or the protein kinase C activators 12-O-tetradecanoylphorbol-13-acetate (TPA) and bryostatin 1. In addition TPA decreased the intracellular GSH content, caused ERK activation, and potentiated the As$_2$O$_3$-provoked activation of p38 and JNK. The addition of N-acetyl-l-cysteine, the PKC inhibitor GF109203X, and the MEK/ERK inhibitors PD98059 and U0126 attenuated both apoptosis induction and GSH decrease, whereas the p38 inhibitor SB203580 and the JNK inhibitor SP600125 were ineffective. TPA also potentiated ERK activation and GSH depletion when added simultaneously to cadmium chloride (CdCl$_2$) and doxorubicin. However, TPA only enhanced apoptosis in the case of CdCl$_2$, which is a GSH-sensitive agent, whereas it reduced the toxicity of doxorubicin and other DNA-specific drugs. Finally, preincubation for 14–24 h with TPA did not potentiate but, instead, attenuated the As$_2$O$_3$- and CdCl$_2$-provoked apoptosis. The same result was obtained by preincubation with bryostatin 1 and other differentiation inducers. It is concluded that TPA increases the apoptotic action of As$_2$O$_3$, an effect mediated by ERK activation and GSH depletion. However, the increase in apoptosis is only effective in non-differentiated cells.

12-O-Tetradecanoylphorbol-13-acetate May Both Potentiate and Decrease the Generation of Apoptosis by the Antileukemic Agent Arsenic Trioxide in Human Promonocytic Cells

REGULATION BY EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASES AND GLUTATHIONE*

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Asengic trioxide (As$_2$O$_3$) has recently attracted great attention because of its capacity to cause complete remission of newly diagnosed and relapsed acute promyelocytic leukemia. In fact, at physiologically tolerable concentrations (<5 μM in plasma), As$_2$O$_3$ readily destroys acute promyelocytic leukemia cells by apoptosis, with a mechanism that involves the degradation of the promyelocytic leukemia-retinoic acid receptor fusion oncoprotein, generally expressed in this type of leukemia (2). Moreover, albeit with lower efficacy, this agent causes apoptosis in other cell types, indicating that it may also be useful for the treatment of other malignancies (2–4). For this reason it is of great interest to analyze the molecular mechanisms responsible for cell death induction by arsenic along with the factors that may modulate (either potentiating or reducing) its toxicity.

One of the most relevant aspects in the regulation of cell death is the signaling of apoptosis by serine/threonine kinases, a broad category of kinases that includes among others the calcium-dependent protein kinases (PKCs) and the mitogen-activated protein kinases (MAPKs). 12-O-Tetradecanoylphorbol-13-acetate (TPA) is a powerful PKC activator that has been commonly reported to inhibit the generation of apoptosis by receptor activation (6–8), growth factor deprivation (9), and different cytotoxic agents (10–13). Nevertheless, this phorbol ester has occasionally been observed to potentiate apoptosis induction (14, 15). Among the three main members that integrate the MAPK family in mammalian cells, the stress-activated protein kinase 1 (c-Jun NH$_2$-terminal kinases (JNKs)) and stress-activated protein kinase 2 (p38) are generally associated to apoptosis induction. By contrast, the extracellular signal-regulated protein kinases (ERK1/2, p44/42) are generally associated to mitogenesis and as such inversely related to apoptosis (5). However, there are also some cases in which the ERKs may exert a pro-apoptotic action (16–19). Of note, the PKC and MAPK pathways are not totally independent. For instance, PKC activates the MEK/ERK pathway via Raf-1 phosphorylation (20, 21), which might account at least in part for the capacity of TPA and other PKC activators to modulate apoptosis.

Some authors have recently examined the capacity of TPA to modulate the toxicity of As$_2$O$_3$ in human myeloid cells, with
Cultures treated for 24 h with 20 nM TPA and As$_2$O$_3$, alone (As) or with TPA (As/TPA). Frequency of apoptotic cells in U-937 cell cultures treated for the indicated time periods with 20 nM TPA or with 4 μM As$_2$O$_3$ alone or with TPA. Cell distribution according to their DNA content as determined by flow cytometry in untreated U-937 cell cultures (Cont) and in cultures treated for 24 h with 20 nM TPA and 4 μM As$_2$O$_3$ alone or in combination. The fraction of cells with sub-G$_1$ DNA content (Ap) are considered as apoptotic. The same experiment as in A using HL-60 promyelocytic cells. In all combined treatments TPA and As$_2$O$_3$ were applied simultaneously. The data in A, B, and C represent the mean ± S.D. of at least three determinations. The histograms in C are representative of one of two determinations, with similar results.

**Fig. 3.** Modulation of As$_2$O$_3$ toxicity by different agents. The histogram shows the frequency of apoptosis in U-937 cell cultures treated for 24 h with 10 nM bryostatin 1 (Bryo), 10 nM VD3, and 0.75 mM sodium butyrate (But) or treated with 4 μM As$_2$O$_3$ with or without the simultaneous addition of the indicated agents. All other conditions were as in Fig. 1.

The As$_2$O$_3$ toxicity, an effect also obtained by preincubation with other differentiation inducers.

**EXPERIMENTAL PROCEDURES**

**Materials**—All components for cell culture were obtained from Invitrogen. Bryostatin 1 and GF109203X were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), cadmium chloride and sodium butyrate were from Merck, 4,6-diamino-2-phenylindole was from Serva (Heidelberg, Germany), and monochlorobimane was from Molecular Probes (Eugene, OR). The kinase inhibitors PD98059, U0126, SB203580, SP600125, and LY294002, the caspase 3-specific substrate N-acetyl-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-VAD-FMK (+Z-VAD)) and the caspase inhibitor benzoyl oxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-Fmk) were obtained from Calbiochem. Rabbit polyclonal antibodies against human p44/42 MAPK, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), p38 MAPK, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), stress-activated protein kinase (SAPK/JNK), and phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-human HSP70 monoclonal antibody (clone C92F3A-5, which specifically recognizes the stress-inducible form of HSP70) was obtained from Stressgen Biotechnologies (Victoria, Canada). Anti-rabbit peroxidase-conjugated antibody was from DAKO Diagnostics, S. A. (Barcelona, Spain). All other reagents were from Sigma.

**Cells and Treatments**—U-937 human promonocytic leukemia cells (25) and HL-60 human promyelocytic leukemia cells (26) were routinely grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum and 0.2% sodium bicarbonate and antibiotics in a non-coincident results. For instance, Sordet et al. (22) indicate that TPA-differentiated cells exhibited an increased susceptibility to apoptosis induction by As$_2$O$_3$, which was associated to a decrease in reduced glutathione (GSH) intracellular content and to the accumulation of reactive oxygen species. However, other authors indicate that the phorbol ester did not affect (23) or even reduce (24) the generation of apoptosis by As$_2$O$_3$. These discrepancies led us to examine the capacity of TPA and some other agents to modulate the apoptotic action of As$_2$O$_3$ and other cytotoxic agents in U-937 promonocytic leukemia cells using different experimental conditions. The obtained results indicated that the generation of apoptosis by the GSH-sensitive agents As$_2$O$_3$ is potentiated by the simultaneous addition of TPA or bryostatin 1. The increase in apoptosis is mediated by ERK activation and seems to be a consequence, at least in part, of GSH depletion. However, after a prolonged preincubation TPA does not potentiate and instead decreases...
Regulation of As$_2$O$_3$ Toxicity by PKC, MAPKs, and GSH

Fig. 4. ERK activation and effect of PKC and ERK inhibitors. A-C, extracts (10 $\mu$g of protein per lane) obtained from U-937 untreated cells (Cont) or cells subjected to the indicated treatments were assayed by Western blot using antibodies which specifically recognize total and phosphorylated ERKs (ERK-tot and ERK-P, respectively). A, cells were treated for the indicated time-periods with 20 nM TPA alone. B, cells were treated for the indicated time periods with 4 nM As$_2$O$_3$ alone and with TPA. C, cells were treated for 18 h with As$_2$O$_3$ plus TPA in the absence (–) or presence of 1 $\mu$M GF109203X (GF), 20 $\mu$M PD98059 (PD), or 2.5 $\mu$M U0126 (U). D-F, frequency of apoptotic cells in U-937 cell cultures treated for 24 h with 4 nM As$_2$O$_3$, either alone or in combination with TPA or bryostatin 1 and in the absence or presence of GF109203X (GF), PD98059 (PD), or U0126 (U) as indicated by chromatin fragmentation (D and F) or reduced (sub-G$_1$) DNA content (E). In F the kinase inhibitors were added at the indicated times in relation to As$_2$O$_3$ plus TPA (here considered as hour zero). In all other experiments the kinase inhibitors were added 1 h before As$_2$O$_3$ plus TPA. All other conditions were as in Figs. 1 and 3.

Measurement of Caspase-3 Activity—Samples of 4 $\times$ 10$^6$ cells were collected by centrifugation, washed twice with ice-cold phosphate-buffered saline, resuspended in 50 $\mu$l of ice-cold lysis buffer (1 mM dithiothreitol, 0.03% Nonidet P-40 (v/v), in 50 mM Tris, pH 7.5), kept on ice for 30 min, and finally centrifuged at 14,000 rpm for 10 min.

Measurement of GSH Levels—The total cellular GSH content was determined by fluorometry after cell loading with monochlorobimane to complete 200 $\mu$l were prepared in triplicate in 96-well microtiter plates and incubated for 1 h at 37°C. The absorption was measured by spectrophotometry at 405 nm.

Determination of Cell Differentiation—Cell differentiation was assessed by measuring the surface expression of CD11b/CD18 and CD11c/CD18 leukocyte integrins. With this aim indirect immunofluorescence assays were carried out using the Bear 1 (anti-CD11b) and HC1/1 (anti-CD11c) monoclonal antibodies in combination with flow cytometry following the previously described procedure (29).

Measurement of CD11b/CD18 expression was determined by fluorometry after cell loading with monochlorobimane following the previously described procedure (28).

Immunoblot Assays—To obtain total cellular protein extracts cells were collected by centrifugation, washed with phosphate-buffered saline, and lysed by 5 min of heating at 100°C followed by sonication in Laemmli buffer containing a protease inhibitor mixture, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. The extracts were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected as previously described (27).

Determination of apoptosis—Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G$_1$ DNA content. To examine chromatin structure, cells were lysed with 4,6-diamidino-2-phenylindole, and examined by fluorescence microscopy. To measure DNA content, cells were permeabilized, stained with propidium iodide, and examined by flow cytometry. These procedures were described in detail elsewhere (27).
RESULTS

Apoptosis Induction—Fig. 1 shows the capacity of TPA and As\textsubscript{2}O\textsubscript{3}, alone and in combination to induce the expression of apoptotic markers in human myeloid cells. As\textsubscript{2}O\textsubscript{3} caused a concentration-dependent (Fig. 1A) and time-dependent (Fig. 1B) increase in the frequency of U-937 promonocytic cells with fragmented chromatin, which is characteristic of apoptosis. TPA (20 nM) was almost innocuous in itself (≈8% of apoptotic cells at 24 h of treatment versus 4% in untreated cells) but greatly potentiated the apoptotic action of As\textsubscript{2}O\textsubscript{3} when both agents were simultaneously applied. The potentiation by TPA of the As\textsubscript{2}O\textsubscript{3}-provoked cell death was confirmed by measuring the frequency of cells with a decreased (sub-G\textsubscript{1}) DNA content, which is also an indicator of apoptosis (Fig. 1C), and was further corroborated by measuring the stimulation of caspase-3 activity (Fig. 2A) and the capacity of the caspase inhibitor Z-VAD-Fmk to inhibit cell death (Fig. 2B). TPA also potentiated the As\textsubscript{2}O\textsubscript{3}-provoked apoptosis in HL-60 human promyelocytic cells (Fig. 1D), showing that the phenomenon is not restricted to the U-937 cell line. On the basis of the results here obtained, the concentration of 4 μM As\textsubscript{2}O\textsubscript{3} was adopted for further experiments except when otherwise indicated.

TPA is a potent PKC activator as well as a differentiation inducer of myeloid cells. For this reason we found it of interest to examine whether the generation of apoptosis by arsenic could be altered by the simultaneous administration of 10 nM VD3 or 0.75 mM sodium butyrate, which in our experiments induced myeloid cell differentiation (28, 30), or 10 nM brystatin 1, which is a PKC activator (albeit with different isoform specificity than TPA) (31) and differentiation inducer (32). The results in Fig. 3 indicate that brystatin 1 potentiated the As\textsubscript{2}O\textsubscript{3}-provoked apoptosis, but VD3 and sodium butyrate were ineffective. This indicates that the stimulatory action of TPA on apoptosis is not related with differentiation induction.

MAPK Activation—As indicated above PKC activation may in turn activate the ERK pathway. For this reason, immunoblot assays were carried out to determine the activation of ERK1/2, as measured by their increased phosphorylation, in cells treated with As\textsubscript{2}O\textsubscript{3} and TPA either alone or in combination. It was found that arsenic alone did not significantly cause ERK activation (Fig. 4B), whereas this kinase was greatly activated by TPA alone (Fig. 4A) and by the combination of As\textsubscript{2}O\textsubscript{3} plus TPA (Fig. 4B). The activation was already observed at 1 h of treatment and still persisted at 24 h.

To analyze whether there is a cause-effect relationship between ERK activation and potentiation of apoptosis by TPA we made use of appropriate pharmacological inhibitors, namely the PKC inhibitor GF109203X (33) and the MEK/ERK inhibitors PD98059 and U0126 (34, 35). Other more direct experimental approaches were not employed due to the poor efficacy of transfection of U-937 cells (result not shown). Control assays indicated that 1 μM GF109203X, 2.5 μM U0126, and 20 μM PD98059 prevented the As\textsubscript{2}O\textsubscript{3} plus TPA-induced ERK phosphorylation (Fig. 4C), proving the efficacy of the inhibitors and confirming that PKC activation in fact precedes and regulates ERK activation. As shown in Fig. 4, D and E, the kinase inhibitors attenuated apoptosis in As\textsubscript{2}O\textsubscript{3} plus TPA-treated cells, indicating that under these experimental conditions ERK activation effectively mediates apoptosis induction. The same result was obtained using brystatin 1 instead of TPA (Fig. 4D).

As demonstrated by kinetic assays, the kinase inhibitors were only effective when applied at the same time as TPA plus As\textsubscript{2}O\textsubscript{3} or shortly thereafter (up to 4 h, approximately) (Fig. 4F).

For comparison we also measured the behavior of p38 and JNK since these kinases may be also activated by TPA in U-937 cells (36). As indicated in Fig. 5A, treatment with As\textsubscript{2}O\textsubscript{3} alone induced the phosphorylation/activation of p38 and JNK, which was potentiated by TPA. The administration of 10 μM SB20358, specific for p38 (37), or 10 μM SP600125, specific for JNK (38), the maximum concentrations that were non-toxic in long term treatments, reduced kinase activation (Fig. 5B) but did not attenuate apoptosis (Fig. 5C). This suggests that p38 and JNK are not primarily responsible for the potentiation by TPA of the As\textsubscript{2}O\textsubscript{3}-provoked apoptosis.

Changes in GSH Content—It has been described that As\textsubscript{2}O\textsubscript{3} is a GSH-sensitive agent, in the sense that its toxicity is enhanced after GSH depletion (39, 40). This was corroborated by us using BSO, a specific inhibitor of γ-glutamylcysteine synthetase activity, the rate-limiting enzyme for GSH biosynthesis (41). In fact BSO reduced the intracellular GSH content (Fig. 6A, left panel) and enhanced the generation of apoptosis by As\textsubscript{2}O\textsubscript{3} (Fig. 6A, right panel). In addition, it was reported that TPA-differentiated myeloid cells exhibited lower GSH content than non-differentiated cells (22). For these reasons, we wanted to measure the alteration of GSH levels in cells treated with As\textsubscript{2}O\textsubscript{3} and TPA alone and in combination in the absence or the presence of the PKC and MAPK inhibitors. For comparison we also measured the GSH content in cells treated with the differentiation inducer VD3. The results, represented in Fig. 6, B–D, were as follows. (i) Treatment with 4 μM As\textsubscript{2}O\textsubscript{3} alone (which as indicated above moderately induced apoptosis but failed to induce ERK activation) did not decrease the GSH content (Fig. 6B). (ii) Treatment with TPA alone (which as...
indicated above did not cause significant apoptosis but induced ERK activation) caused GSH depletion (Fig. 6B). Of note, no GSH decrease was observed in cells treated for 24–72 h with VD3 (Fig. 6B) although at 72 h of treatment the cells expressed differentiation markers (results not shown). (iii) The combination of As$_2$O$_3$ plus TPA (which as indicated above greatly induced apoptosis as well as ERK activation) caused a greater GSH decrease than TPA alone (Fig. 6B). (iv) The GSH depletion caused by TPA alone or by As$_2$O$_3$ plus TPA was attenuated by the PKC and ERK inhibitors (which as indicated above attenuated apoptosis) but was not affected by the p38 and JNK inhibitors (which also failed to prevent apoptosis) (Fig. 6C). Similar results were obtained using bryostatin 1 instead of TPA (Fig. 6C). And (v) the administration of PD98059 7 h after As$_2$O$_3$ plus TPA was unable to restore the GSH level (Fig. 6D), which is congruent with the above-inferred inability of the ERK inhibitor to prevent apoptosis at this time of treatment. Taken together these results indicate the existence of a strict correlation between ERK activation, GSH depletion, and potentiation of apoptosis in As$_2$O$_3$ plus TPA-treated cells. Moreover, they suggest that GSH depletion is not a mere consequence of cell death or differentiation induction.

Finally, experiments were carried out using NAC, a GSH-increasing agent earlier employed by other authors in combination with As$_2$O$_3$ (39, 42). We found that the administration of 10 mM NAC did not prevent ERK activation (result not shown) but restored the GSH content (Fig. 6E, left panel) and reduced apoptosis (Fig. 6E, right panel) in As$_2$O$_3$ plus TPA-treated cells. Of note, the possibility that such attenuation of apoptosis could be due to arsenic scavenging by NAC may be ruled out since NAC did not reduce the toxicity of As$_2$O$_3$ plus PD98059, an inhibitor of the phosphatidylinositol 3-kinase pathway that potentiated the As$_2$O$_3$-provoked apoptosis (Fig. 6E, right panel), and NAC also failed to prevent the As$_2$O$_3$-provoked activation of HSP70 expression, a stress-inducible protein (Fig. 6F). These results, which are fully consistent with earlier observations (39, 42), support the existence of a cause-effect relationship between GSH depletion and potentiation of apoptosis in our experimental model.

Effects of Other Cytotoxic Drugs—The preceding results strongly suggest that the potentiation of As$_2$O$_3$-provoked apoptosis by TPA could be the consequence, at least in part, of GSH depletion. If this was the case we could predict a similar potentiation of apoptosis when the phorbol ester is used in combination with other GSH-sensitive agents but probably not with GSH-insensitive drugs. Earlier observations indicated that GSH depletion enhanced the toxicity of the heavy metal cadmium (43, 44) but did not affect the toxicity of anti-DNA topoisomerase drugs in myeloid cells (29, 45). Hence, we decided to compare the effect of BSO and TPA on U-937 cells treated with cadmium chloride (CdCl$_2$, 40 $\mu$M) and with the antitumor anti-DNA topoisomerase drugs etoposide (0.5 $\mu$M), camptothecin (50 nM), and doxorubicin (0.5 $\mu$M). As expected, BSO potentiated apoptosis in the case of CdCl$_2$ but not in the case of the anti-topoisomerase drugs (Fig. 7A), and according to our prediction, TPA only potentiated apoptosis in the case of CdCl$_2$ (Fig. 7B). Indeed, TPA reduced the toxicity of the anti-topoisomerase drugs (Fig. 7B), which is consistent with the commonly reported protective action of the phorbol ester (6–13).

Of note, the different effect of TPA on the toxicity of CdCl$_2$ and anti-topoisomerase drugs may not be attributed to a different behavior of ERKs or GSH. In fact, TPA potentiated ERK activation (Fig. 7C) and exacerbated GSH depletion (Fig. 7D) by both CdCl$_2$ and doxorubicin. Moreover, GP109203X and PD98059 restored the GSH level in both CdCl$_2$ plus TPA- and doxorubicin plus TPA-treated cells (Fig. 7D). However, although the kinase inhibitors attenuated apoptosis induction by CdCl$_2$ plus TPA (as in the case of As$_2$O$_3$ plus TPA), they did not attenuate and even increased apoptosis in the case of doxoru-
bicin plus TPA (Fig. 7E). Taken together, these results corroborate the conclusion that the unusual pro-apoptotic action of TPA and ERKs in the case of As$_2$O$_3$ is due to the GSH sensitivity of this agent.

**Effect of Preincubation with TPA**—In all the preceding experiments, TPA was administered simultaneously to As$_2$O$_3$ or the other cytotoxic drugs. Hence, new experiments were carried out in which the cells were preincubated for different times with the phorbol ester before treatment with As$_2$O$_3$. Some of the obtained results are represented in Fig. 8. Preincubation with TPA for up to 6 h was still compatible with a potentiation of apoptosis. However, preincubation for 14–24 h, a treatment period that suffices to induce the expression of differentiation markers (46, and results not shown), did not potentiate and, instead, attenuated the As$_2$O$_3$-provoked apoptosis (Fig. 8, A and B) and, accordingly, decreased caspase-3 activity (Fig. 8C). The decrease in apoptosis occurred despite the reduced GSH level, which remained below 40% of control value during the whole period of As$_2$O$_3$ treatment (result not shown). Using this experimental design, similar results were obtained when the cells were treated with CdCl$_2$ or anti-topoisomerase drugs instead of As$_2$O$_3$ (Fig. 8D) or when they were preincubated for 24 h with bryostatin 1 or for 48 h with the differentiation inducers VD3 and sodium butyrate instead of TPA (Fig. 8E).

**DISCUSSION**

The results in this work corroborate earlier observations indicating that As$_2$O$_3$ and CdCl$_2$ cause death by apoptosis in myeloid leukemia cells and that the toxicity of these agents is exacerbated by GSH depletion (39, 44). The increase in toxicity may be manifested as an increase in the frequency of apoptosis (Refs. 39 and 40 and results in this work) or even as a change in the mode of death from apoptosis to necrosis (44), depending on the experimental conditions. By contrast, the toxicity of anti-DNA topoisomerase drugs was apparently insensitive to GSH depletion, a result also consistent with earlier observations in the myeloid cell model (29, 45). The dependence of As$_2$O$_3$ and CdCl$_2$ toxicity on GSH content may be explained by the capacity of arsenic and cadmium ions to directly react with GSH (43, 47). Thus, GSH depletion may lead to a decrease in GSH-metal interactions, increasing the intracellular concentration of free arsenic and cadmium ions and, hence, their toxicity. In particular, the increase in metal ion concentration could exacerbate mitochondrial dysfunction, since it is known that arsenic and cadmium directly target the mitochondria (48, 49). In addition, GSH depletion might potentiate cell death by facilitating the accumulation of reactive oxygen species due to the reduction in glutathione peroxidase activity (50). Nevertheless, the relevance of this mechanism in the present experiments is questionable, since treatment with low As$_2$O$_3$ concentrations did not produce a detectable increase in peroxide levels in the U-937 cell system (51).

As indicated above, TPA was reported to potentiate (22) and reduce (24) the generation of apoptosis by As$_2$O$_3$ in myeloid leukemia cells. Our present results indicate that both types of response are possible, depending on the conditions of treatment. Thus, TPA potentiated apoptosis when applied simultaneously to, or shortly before As$_2$O$_3$ and CdCl$_2$ but decreased apoptosis after a prolonged (14–24 h) incubation period. This contrasts with the response of anti-topoisomerase drugs, the toxicity of which was always reduced by TPA independently of...
the conditions of treatment. The potentiation of As$_2$O$_3$ toxicity by TPA was mediated by PKC activation, since the same result was obtained using bryostatin 1 instead of TPA, and apoptosis was attenuated by the PKC inhibitor GF109203X. The action of TPA was in turn mediated by PKC activation, since GF109203X prevented the TPA plus As$_2$O$_3$-provoked ERK phosphorylation, and the potentiation of apoptosis by TPA or bryostatin 1 was reduced by the ERK inhibitors U0126 and PD98059. This infrequent pro-apoptotic effect of ERKs, normally considered as a survival-inducing kinase, contrasts with the apparent irrelevance of p38 and JNK, normally considered as pro-apoptotic kinases. In fact, TPA potentiated the activation of p38 and JNK, but SB203580 and SP600125 failed to reduce the TPA plus As$_2$O$_3$-provoked apoptosis. Concerning the reduction of As$_2$O$_3$ and CdCl$_2$ toxicity after a prolonged preincubation with TPA, we may reasonably suppose that this is a consequence of differentiation. In fact, U-937 cells treated with TPA for 14–24 h already express differentiation markers, and the toxicity of As$_2$O$_3$ was also decreased by preincubation with other differentiation inducers, namely bryostatin 1, sodium butyrate, and VD3. The resistance of differentiated myeloid cells to apoptosis induction by diverse cytotoxic agents has been also documented by other authors (11, 52–54). Because differentiation and apoptosis are alternative, mutually excluding pathways (55), a restraint in apoptosis may be expected once the differentiation program has been executed.

Finally, the present results indicate that GSH depletion is a key factor linking ERK activation and potentiation of apoptosis in As$_2$O$_3$ plus TPA-treated cells. This conclusion is supported by the concurrence of multiple evidences. In fact (i) TPA-mediated ERK activation always correlated with GSH decrease (Fig. 4A and B, versus Fig. 6B and Fig. 7C versus D) no matter the effect of TPA on apoptosis, and the administration of ERK inhibitors restored the GSH content (Figs. 6C and 7D). Moreover, treatment with As$_2$O$_3$ alone did not cause ERK activation nor GSH decrease (Fig. 4B versus Fig. 6B). (ii) The apoptotic action of As$_2$O$_3$ was potentiated after GSH depletion by BSO and TPA, and this potentiation was abrogated when the GSH level was restored by NAC (Fig. 6). (iii) Treatment with TPA alone caused GSH decrease without significant apoptosis (Fig. 6B versus Fig. 1), indicating that GSH depletion is not a mere consequence of cell death in this type of experiment. And (iv) finally, TPA-mediated ERK activation and GSH depletion correlated with apoptosis increase only in the case of GSH-sensitive agents such as As$_2$O$_3$ and CdCl$_2$. By contrast, TPA and ERKs played their canonical, anti-apoptotic role when used with GSH-insensitive, anti-DNA topoisomerase drugs (Fig. 7). Hence, we may reasonably conclude that ERK activation leads to GSH depletion, which in turn accounts at least in part for the increased apoptosis in TPA plus As$_2$O$_3$-treated cells. Experiments are in course to determine the mechanism(s) responsible for GSH depletion, i.e. synthesis inhibition or accelerated loss. In addition, it remains to be investigated whether the conclusions here obtained may be extended to other antitumor drugs, e.g. cisplatin, a GSH-sensitive drug (29), the toxicity of which may be potentiated by ERK activation (18).

### References

1. Léonard, A. (1991) in Metals and Their Compounds in the Environment. Occurrence, Analysis, and Biological Relevance (Merian, E., ed) pp. 751–774, VCH Publishers, Inc., New York.
2. Miller W. H., Jr., Schipper, H. M., Lee, J. S., Singer, J., and Waxman, S. (2002) *Cancer Res.* 62, 3893–3903.
3. Murgo, A. J. (2001) *Oncologist* 6, Suppl. 2, 22–28.
4. Wang, Z. Y. (2001) *Cancer Chemother. Pharmacol.* 48, Suppl. 1, 72–76.
5. Cross, T. G., Schell-Toellner, L., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) *Exp. Cell Res.* 256, 34–41.
6. Sarker, M., Ruiz-Ruiz, C., and López-Rivas, A. (2001) *Cell Death Differ.* 8, 172–181.
7. Herrant, M., Luciano, F., Loubat, A., and Auberger, P. (2002) *Oncogene* 21, 4957–4968.
8. Söderén, T. S., Poukkula, M., Holmström, T. H., Heiskanen, K. M., and...
Regulation of As$_2$O$_3$ Toxicity by PKC, MAPKs, and GSH

Eriksson, J. E. (2002) J. Immunol. 169, 2851–2860
9. Behrens, M. M., Strasser, U., Koh, J. Y., Owg, B. J., and Choi, D. W. (1999) Neuroscience 94, 917–927
10. Messmer, U. K., and Brune, B. (1997) Br. J. Pharmacol. 121, 625–634
11. Sordet, O., Betteaud, A., Bruey, J. M., Eymun, B., Drion, N., Ivenson, M., Garrido, C., and Solary, E. (1999) Cell Death Differ. 6, 351–361
12. Zhuang, S., Demirs, J. T., and Koochev, I. E. (2001) Oncogene 20, 6764–6776
13. Shonai, T., Adachi, M., Sakata, K., Takekawa, M., Endo, K., Imai, K., and Hareyama, M. (2002) Cell Death Differ. 9, 963–971
14. Jun, C. D., Pae, H. H., Kwak, H. J., Yoo, J. C., Choi, B. M., Oh, C. D., Chun, J. S., Park, S. G., Park, Y. H., and Chung, H. T. (1999) Cell. Immunol. 194, 36–46
15. Cartee, L., Smith, R., Dai, Y., Rahmani, M., Rosate, R., Almenara, J., Dent, P., and Grant, S. (2002) Mol. Pharmacol. 61, 1313–1321
16. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) J. Biol. Chem. 272, 20490–20494
17. Bhat, N. R., and Zhang, P. (1999) J. Neurochem. 72, 112–119
18. Wang, X., Martindale, J. L., and Holbrook, N. J. (2000) J. Biol. Chem. 275, 39435–39443
19. Vrana, J. A., and Grant, S. (2001) Blood 97, 2105–2114
20. Carroll, M. F., and May, W. S. (1994) J. Biol. Chem. 269, 1249–1256
21. Marquardt, B., Frith, D., and Stabel, S. (1994) Oncogene 9, 3213–3218
22. Sordet, O., Rebé, C., Leroy, I., Bruey, J. M., Garrido, C., Miguez, C., Lizard, G., Pleschette, S., Corcos, L., and Solary, E. (2001) Blood 97, 3901–3904
23. Huang, X. J., Wiernick, P. H., Klein, R. S., and Gallagher, B. E. (1999) Med. Oncol. 16, 58–64
24. Iwama, K., Nakajo, S., Aizaki, T., and Nakaya, K. (2001) Int. J. Cancer 92, 518–526
25. Sundstrom, C., and Nilsson, K. (1976) Int. J. Cancer 17, 565–577
26. Collins, S., Gallis, R., and Gallion, R. (1977) Nature 270, 347–349
27. Galan, A., Garcia-Bermejo, M. L., Troyano, A., Vilaboa, N. E., De Blas, E., Kanzietz, M. G., and Aller, P. (2000) J. Biol. Chem. 275, 11418–11424
28. Rius, C., Cabanas, C., and Aller, P. (1990) Exp. Cell Res. 188, 129–134
29. Troyano, A., Fernández, C., Sancho, P., De Blas, E., and Aller, P. (2001) J. Biol. Chem. 276, 47107–47115
30. Rius, C., and Aller, P. (1988) Cell Differ. Dev. 28, 39–46
31. Kraft, A. S., Smith, J. B., and Berkow, R. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1334–1338
32. Vrana, J. A., Saunders, A. M., Chellappan, S. P., and Grant, S. (1998) Differentiation 63, 35–42
33. Toullec, D., Panetti, P., Coste, H., Beliveau, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Bourrie, E., Loriselle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
34. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689
35. Pavata, M. F., Horiechi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feerer, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Mapolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) J. Biol. Chem. 273, 18623–18632
36. Franklin, C. C., and Kraft, A. S. (1997) J. Biol. Chem. 272, 18917–18923
37. Franz, B., Klatt, T., Pangs, P., Parsee, J., Rolando, A., Williams, H., Ticci, M. J., O’Keefe, S. J., and O’Neill, E. A. (1998) Biochemistry 37, 13846–13853
38. Han, Z., Boyle, D. L., Chang, L., Bennet, B., Karin, M., Yang, L., Manning, A. M., and Firestein, G. S. (2001) J. Clin. Invest. 108, 75–81
39. Dai, B., Weinberg, R. S., Waxman, S., and Jing, Y. (1999) Blood 93, 268–277
40. Cai, X., Shen, Y. L., Zhu, Q., Jia, P. M., Zhou, L., Huang, Y., Zhang, J. W., Xiong, S. M., Chen, S. J., Wang, Z. Y., Chen, Z., and Chen, G. Q. (2000) Leukemia (Baltimore) 14, 262–270
41. Griffith, O. W., and Meister, A. (1979) J. Biol. Chem. 254, 7558–7560
42. Hu, X. M., Hirano, T., and Oka, K. (2003) Cancer Chemother. Pharmacol. 52, 47–58
43. Ochi, T., Oesaka, F., Takahashi, K., and Ohsawa, M. (1988) Chem. Biol. Interact. 65, 1–14
44. Galan, A., Troyano, A., Vilaboa, N. E., Fernández, C., De Blas, E., and Aller, P. (2001) Biochim. Biophys. Acta 1538, 34–46
45. Fernandes, R. S., and Cotter, T. G. (1994) Biochem. Pharmacol. 48, 675–681
46. Rius, C., and Aller, P. (1992) J. Cell Sci. 101, 395–401
47. Scott, N., Hatlelid, K. M., Mackenzie, N. E., and Carter, D. E. (1993) Chem. Res. Toxicol. 6, 102–106
48. Larochette, N., Desaun, D., Jacotet, E., Brenner, C., Marro, I., Susin, S. A., Zamzami, N., Xie, Z., Reed, J., and Kroemer, G. (1999) Exp. Cell Res. 249, 413–421
49. Belyaeva, E. A., Glazunov, V. V., and Kuretkov, S. M. (2002) Arch. Biochem. Biophys. 405, 252–264
50. Dickinson, D. A., and Froman, H. J. (2002) Biochem. Pharmacol. 64, 1019–1026
51. Jing, B. Y., Dai, J., Chalmers-Redman, R. M., Tatton, W. G., and Waxman, S. (1999) Blood 94, 2102–2111
52. Solary, E., Bertrand, R., Kohn, K. W., and Pummier, Y. (1993) Blood 81, 1359–1368
53. Xu, H. M., Tepper, C. G., Jones, J. B., Fernandez, C. E., and Studzinski, G. P. (1993) Exp. Cell Res. 209, 369–374
54. Shiiki, K., Yoshikawa, H., Kinoshita, H., Takeda, M., Ueno, A., Nakajima, Y., and Takada, K. (2000) Cell Death Differ. 7, 939–940
55. Hass, R. (1994) Crit. Rev. Oncog. 5, 359–371
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