Blockage of Multidrug Resistance-Associated Proteins Potentiates the Inhibitory Effects of Arsenic Trioxide on CYP1A1 Induction by Polycyclic Aromatic Hydrocarbons

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ABSTRACT
Arsenic is a toxic metalloid known to interact with drug-metabolizing enzymes. In the present study, we investigated the effects of arsenic trioxide (As$_2$O$_3$), recently used as an antican- cer drug, on the expression of human cytochrome P450 (P450) 1A1, which bioactivates polycyclic aromatic hydrocarbons into mutagenic metabolites. Clinically relevant concentrations (0.25–5 μM) of As$_2$O$_3$ were demonstrated to inhibit CYP1A activity in primary human hepatocytes and hepatoma Hep3B and HepG2 cells coexposed to 3-methylcholanthrene (3MC), benzo(a)pyrene, or dioxin and the metalloid for 24 h. Inhibition reached 50 and 90% in Hep3B cells treated with 1 and 5 μM As$_2$O$_3$, respectively, and was not due to direct interaction of the metalloid with CYP1A1. As$_2$O$_3$ (2.5–5 μM) was demonstrated to markedly reduce induction of CYP1A1 mRNA and apoprotein levels and gene promotor activity in 3CM-treated Hep3B cells, whereas lower concentrations (0.25–1 μM) were ineffective. These effects of As$_2$O$_3$ were abrogated by N-acetylcysteine. Surprisingly, this agent was found 1) to block cellular arsenic uptake when coincubated with the metalloid and 2) to increase arsenic efflux through multidrug resistance-associated proteins. In addition, blockade of these transporters was shown to enhance intracellular amounts of metalloid and to potentiate its effects on CYP1A1 gene. Finally, our results have demonstrated that As$_2$O$_3$, at low concentrations routinely reached in As$_2$O$_3$-treated patients, prevents induction of human CYP1A1 gene expression and that such an effect is increased by blocking multidrug resistance-associated proteins.

In spite of its toxicity, arsenic has been used for many centuries as a therapeutic agent against various pathologies, including inflammatory and protozoal diseases (Waxman and Anderson, 2001). More recently, arsenic trioxide (As$_2$O$_3$), a trivalent inorganic form, was found to possess marked antitumoral properties toward hematological malignancies. Different clinical trials have reported that low concentrations of As$_2$O$_3$ were clinically effective in the treatment of acute promyelocytic leukemia and, particularly, in patients who relapsed after conventional therapy (Murgo, 2001). In addition, experimental studies have demonstrated that low concentrations of As$_2$O$_3$ were toxic toward other malignant hematological cells and several solid tumor cell lines (Murgo, 2001). Anticancerous actions of As$_2$O$_3$ are complex; they involved partial cytodifferentiation, inhibition of cell proliferation, induction of apoptosis, and inhibition of angiogenesis (Waxman and Anderson, 2001). As demonstrated for various anticancerous agents, a rapid development of clinical resistance to As$_2$O$_3$ could also been observed in some patients (Soignet et al., 1998) and it was suggested to be associated with increased activity of the multidrug resistance-associated protein 1 (MRP1) in tumor cells (Vernhet et al., 2001a). This transporter was recently shown to be highly expressed in some tumors and to mediate extracellular export of different metalloids, including arsenic (Hipfner et al., 1999; Vernhet et al., 1999).

Cellular effects of arsenic, including apoptosis, are likely linked to oxidative stress (Waxman and Anderson, 2001). Arsenic has been shown to alter cellular redox status and to modify expression of various stress-related genes through production of reactive oxygen species (ROS), depletion of cellular glutathione, or binding to cysteine residues in proteins (Del Razo et al., 2001; Liu et al., 2001). Interestingly,

ABBREVIATIONS: MRP1, multidrug resistance-associated protein 1; ROS, reactive oxygen species; MRP2, multidrug resistance-associated protein 2; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; Ah receptor, aryl hydrocarbon receptor; XRE, xenobiotic responsive elements; NAC, N-acetylcysteine; 3MC, 3-methylcholanthrene; SV40, simian virus 40; EROD, ethoxyresorufin O-deethylase; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate.
the metalloid also regulates expression of genes encoding xenobiotic-metabolizing enzymes (Bergelson et al., 1994; Liu et al., 2001). As diverse redox active compounds, it induces expression of NADP(H):quinone reductase and glutathione S-transferases at the transcriptional level through activation of the antioxidant response element, a cis-acting element present in promoters of these genes (Bergelson et al., 1994). More recently, we demonstrated that arsenic also increased levels of the multidrug resistance-associated protein 2 (MRP2) in rat and human primary hepatocytes (Vernhet et al., 2001b). MRP2 is known to be responsive not only to oxidative stress (Payen et al., 2001) but also to chemicals that activate pregnane X receptor and constitutive androstane receptor pathways (Kast et al., 2002). As with MRP1, this transporter mediates arsenic efflux (Kala et al., 2000). In addition, arsenic can negatively regulate expression of different cytochromes P450 (P450), including CYP1A1 (Jacobs et al., 1999; Vakharia et al., 2001a,b).

CYP1A1 is a major phase I enzyme involved in bioactivation of polycyclic aromatic hydrocarbons (PAHs) into muta-
MRP Blockage Increases Inhibitory Effects of As$_2$O$_3$ on CYP1A1

Figure 2. Direct effects of As$_2$O$_3$, and $\alpha$-naphthoflavone on EROD activity. Hep3B cells, first treated with 5 $\mu$M 3MC for 24 h, and microsomes, prepared from 3MC-treated Hep3B, were incubated without or with 5 $\mu$M As$_2$O$_3$ or with 20 $\mu$M $\alpha$-naphthoflavone (NF) during EROD activity measurement, as described under Materials and Methods.

Materials and Methods

Chemicals. As$_2$O$_3$, $N$-acetylcycteine (NAC), 3-methylcholanthrene (3MC), benzo[\(a\)]pyrene, resveratrol, hydrogen peroxide (H$_2$O$_2$), and $\alpha$-naphthoflavone were supplied from Sigma-Aldrich (St. Louis, MO). Dioxin was obtained from Cambridge Isotope Laboratories (Cambridge, MA). MK571, a leukotriene D4 receptor antagonist (Jones et al., 1989) known to inhibit MRPL and MRP2 activities (Lorusso et al., 2002), was kindly provided by Dr. Ford-Hutchinson (Merck-Front Inc., Quebec, QC, Canada).

Cell Culture Treatments. Human hepatoma Hep3B and HepG2 cells were cultured in Williams' E medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 10 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml hydrocortisone. For all experiments, confluent cellular monolayers were used. Normal human hepatocytes were obtained after collagenase disruption of liver fragments of adult donors undergoing resection for primary or secondary tumors and then cultured as described previously (Vernhet et al., 2001b). All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee.

Arsenic stock solution (5 mM) was obtained by dissolving As$_2$O$_3$ in 0.2 M NaOH. NAC was dissolved in distilled water and adjusted for pH 7.4 with NaOH. In some experiments, cells were first pretreated with 20 mM NAC for at least 6 h; culture medium was next replaced by fresh medium containing NAC, and the cells were treated as indicated. In other experiments, all products were added simultaneously to cell cultures. 3MC, benzo[\(a\)]pyrene, resveratrol, and $\alpha$-naphthoflavone were dissolved in dimethyl sulfoxide, and the final concentration of the solvent in culture medium did not exceed 0.2% (v/v).

Cell Viability and Protein Synthesis. Cytotoxic effects of As$_2$O$_3$ were measured after a 24-h incubation with Hep3B cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay, as described previously (Vernhet et al., 2001a). To test its effects on protein synthesis, Hep3B cells were first treated with As$_2$O$_3$ for 24 h and then pulsed for 90 min with 0.5 $\mu$Ci of $[^{3}H]$leucine (Amersham Biosciences, Piscataway, NJ). Cells were lysed and proteins were precipitated with 30% trichloroacetic acid overnight at 4°C. Radioactive proteins were dissolved by formic acid.

RNA Isolation and Blot Analysis. Total RNAs were extracted from Hep3B cells with SV Total RNA isolation kit (Promega, Madison, WI) as described in the manufacturer's protocol. Ten micrograms of RNA samples was subjected to electrophoresis under denaturing conditions as described previously (Vernhet et al., 2001b). Human CYP1A1 mRNAs were detected using a specific cDNA probe (gift from Dr. de Waziers, Institut National de la Santé et de la Recherche Médicale U490, Paris, France), whereas 18S ribosomal RNAs were analyzed using an 18S ribosomal RNA oligonucleotide.
The presence of increasing concentrations of \( \text{As}_2\text{O}_3 \), for 24 h. Total mRNAs were incubated for another 18 h at which time they were treated for

**Preparation of Microsomal Fractions.** Cells were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA and microsomes were prepared as described previously (Le Ferrec et al., 2002) and stored at \(-80^\circ\text{C}\) in 0.1 M phosphate-buffered saline, pH 7.4, containing 10% glycerol. Protein concentration was determined according to the Bradford (1976) method.

**Western Blot Immunoassays.** Hep3B microsomal proteins, and proteins from whole Hep3B cell lysates prepared as described previously (Payen et al., 2000), were separated on a 10% SDS polyacrylamide gel and then transferred onto nitrocellulose membranes. After blocking, membranes were incubated with a goat anti-human CYP1A1/A2 antibody (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan) or with either the mice anti-human MRP1 monoclonal antibody MRPm6 (Monosan) or the rabbit anti-human MRP2 monoclonal antibody MIII-6 (kindly provided by Dr. Scheppe, Free University Hospital, Amsterdam, The Netherlands). Peroxidase-conjugated antibodies were used as secondary antibodies and blots were developed by chemiluminescence.

**Ethyoxresorufin O-Deethylase (EROD) Activity Assay.** EROD activity, mainly supported by CYP1A1 and CYP1A2 in human cells, was measured on Hep3B microsomes and living cells as described previously by Le Ferrec et al. (2002). Kinetic reading with a SpectraMax Gemini spectrofluorometer (Molecular Devices Sunnyvale, CA) was performed over 30 min.

**Determination of ROS Production.** Cellular production of ROS was analyzed using 2,7’-dichlorodihydrofluorescein diacetate (DCPH-DA), a cell-permeant fluorescent probe. Hep3B cells were labeled for 1 h with 1 \( \mu \text{M} \) DCFH-DA and then incubated with 5 \( \mu \text{M} \) 3MC in the absence or presence of \( \text{As}_2\text{O}_3 \), or \( \text{H}_2\text{O}_2 \) for 24 h. Then, cells were analyzed by the SpectraMax Gemini spectrofluorometer; excitation and emission wavelengths were 485 and 538 nm, respectively. ROS amounts were normalized to cellular protein contents.

**Measurement of Cellular Arsenic Contents.** Cellular arsenic contents were determined after both incubation of cells with 10 \( \mu \text{M} \) \( \text{As}_2\text{O}_3 \) for 3 h (accumulation studies) and reincubation of \( \text{As}_2\text{O}_3 \)-loaded cells in \( \text{As}_2\text{O}_3 \)-free culture medium for up to 3 h (efflux studies). Cellular arsenic contents were quantified using a Zeeman atomic absorption spectrometer (Spectra A300; Varian Medical Systems, Palo Alto, CA) and were normalized to cellular protein content.

**Statistical Analysis.** The results are presented as means ± S.E.M. of at least three independent experiments. Significant differences between means were evaluated with the multirange Dunnett’s \( t \) test. For the variations of relative EROD activity measured in Hep3B cells cultured in the absence or presence of MK571, a two-way analysis of variance followed by a Student’s \( t \) test was used. Criterion of significance of the difference between means was \( p < 0.05 \).

**Results**

Toxic concentrations of \( \text{As}_2\text{O}_3 \) were first determined in Hep3B cells using 3-(4-(5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and measurements of protein synthesis. \( \text{As}_2\text{O}_3 \) (0.25–5 \( \mu \text{M} \) altered neither cell viability nor protein synthesis, whereas higher concentrations markedly reduced both parameters (data not shown). Consequently, only concentrations up to 5 \( \mu \text{M} \) \( \text{As}_2\text{O}_3 \) were used in the following experiments.

To study its effects on CYP1A1 expression, Hep3B and HepG2 cells were incubated simultaneously with 3MC and various concentrations of \( \text{As}_2\text{O}_3 \) for 24 h, before measurement of EROD activity. In these conditions, EROD activity was demonstrated to result only from PAH-induced CYP1A1 expression in human hepatoma cells (Vakharia et al., 2001). As shown in Fig. 1A, \( \text{As}_2\text{O}_3 \) markedly inhibited 3MC-induced

![Graph](image-url)
CYP1A1 activity in Hep3B cells (Fig. 1A). A significant inhibitory effect was detected with a concentration as low as 0.25 μM, and almost total inhibition was obtained with 5 μM As₂O₃. Similar dose-dependent inhibitions of 3MC-induced EROD activity by As₂O₃ were observed in HepG2 cells and human primary hepatocytes (Fig. 1A). The metalloid was also shown to strongly decrease CYP1A1 activity induced by benzo(a)pyrene or dioxin in Hep3B cells (Fig. 1B). In all these experiments, 1 μM As₂O₃ decreased activity by nearly 50%. Basal EROD activity, which mainly results from CYP1A2

Fig. 4. Effects of As₂O₃ on promoter activity in transiently transfected Hep3B cells. A, Hep3B cells were transiently transfected with the p1A1-FL construct and then either untreated or treated with 5 μM 3MC, in the absence or presence of increasing concentrations of As₂O₃, for 24 h. B, Hep3B cells were transiently transfected with the pGL3-XRE3 and then either untreated or treated with 5 μM 3MC, in the absence or presence of 5 μM As₂O₃ or 50 μM resveratrol (res), for 24 h. Luciferase activity was normalized to Renilla luciferase activity. *, p < 0.05.
expression, was not detectable in Hep3B cells and was not modified by As$_2$O$_3$ in primary human hepatocytes (data not shown). To determine whether As$_2$O$_3$ could directly interact with the enzyme, we examined the effects of As$_2$O$_3$ after a 24-h treatment of Hep3B cells with 3MC. As shown in Fig. 2, 5 µM As$_2$O$_3$ modifies CYP1A1 activity neither in 3MC-treated cells nor in microsomes extracted from these cells. In contrast, 20 µM α-naphthoflavone, a direct CYP1A inhibitor (Grafstrom and Stohs, 1981), almost totally blocked EROD activity.

We further examined CYP1A1 gene expression using Northern blot analysis. Figure 3 indicates that simultaneous treatment of Hep3B cells with 3MC and As$_2$O$_3$ markedly altered CYP1A1 mRNA levels. Although low concentrations had only minor effects, 2.5 to 5 µM As$_2$O$_3$ strongly reduced induction of CYP1A1 mRNA levels. To determine whether As$_2$O$_3$ could act at transcriptional level, we next performed transient transfections with p1A1-FL construct, which contains the CYP1A1 gene 5'-flanking region upstream of the luciferase gene. Figure 4A shows that 2.5 and 5 µM As$_2$O$_3$ decreased 3MC-induced luciferase activity by 30 and 40%, respectively. In addition, to examine the effects of As$_2$O$_3$ on Ah receptor pathway, Hep3B cells were transiently transfectioned with the pGL3-XRE3 construct, which contains only three XRE sequences. In these conditions, the metalloid slightly reduced luciferase activity, whereas 50 µM resveratrol, a known Ah receptor antagonist (Ciolino et al., 1998), markedly inhibited luciferase activity in 3MC-treated cells transfected with either pGL3-XRE3 or p1A1-FL(1566) constructs (Fig. 4B). This figure also shows that As$_2$O$_3$ had no effect on basal luciferase activity in untreated Hep3B cells.

In the next set of experiments, we examined the expression of CYP1A1 apoprotein in microsomes prepared from Hep3B cells simultaneously treated with 3MC and As$_2$O$_3$. Western blot analysis showed that 2.5 and 5 µM As$_2$O$_3$ strongly decreased levels of the apoprotein, whereas lower concentrations had no detectable effects (Fig. 5A). However, measurements of EROD activity in microsomes confirmed that As$_2$O$_3$ dose dependently inhibited activity of the CYP1A1 holopro-
Fig. 6. Effects of NAC on ROS production (A) and EROD activity (B) in Hep3B cells. A, untreated and NAC-treated Hep3B cells were first incubated 1 h with 1 μM DCFH-DA and then treated with 5 μM 3MC alone, 5 μM 3MC and As$_2$O$_3$, or with 5 μM 3MC and 1 mM $H_2$O$_2$, in the absence (■) or presence of 20 mM NAC (□), for 24 h. B, cells were treated as described in A except that DCFH-DA was not added. ROS production and EROD activity were then determined as described under Materials and Methods. *, p < 0.05.
Hep3B cells. We first compared intracellular accumulation of arsenic in the absence or presence of NAC, as described above. Our results showed that arsenic amounts measured after a 3-h incubation with As$_2$O$_3$ were 34 ± 1.5 and 1.8 ± 0.3 ng/mg of protein in untreated and NAC-treated Hep3B cells, respectively (Fig. 7A). This marked alteration of cellular metalloid accumulation could result either from a reduced uptake or from an increased efflux of arsenic from Hep3B cells. To test the effect of NAC on efflux, cells were loaded with As$_2$O$_3$ in the absence of NAC and then incubated in arsenic-free medium in the presence of 20 mM NAC. As shown in Fig. 7B, NAC significantly accelerated arsenic efflux because the amounts of metalloid remaining, after a 3-h incubation in arsenic-free medium, were 10-fold lower in NAC-treated cells than those measured in untreated cells. We also examined the effects of MK571, a potent inhibitor of MRP1 and MRP2 efflux pumps, which are thought to mediate arsenic efflux from human cells (Kalå et al., 2000; Vernhet et al., 2001a). Figure 7B indicates that the remaining amounts of metalloid were significantly higher in cells treated with MK571 or MK571 and NAC than those measured in untreated cells or treated with NAC, respectively, thereby suggesting that blockade of a MK571-sensitive pump had lowered arsenic efflux. By Western blotting analysis, both MRP1 and MRP2 proteins were found to be highly expressed in Hep3B cells (data not shown). To ensure that NAC effect on efflux also mediated the decrease of metalloid intracellular accumulation, hepatoma cells were directly incubated simultaneously with 3MC, As$_2$O$_3$ and MK571 (Fig. 8); in contrast, MK571 alone had no effect on EROD activity measured in 3MC-treated Hep3B cells exposed to 3MC + MK571 (Fig. 8); in contrast, MK571 alone had no effect on EROD activity measured in 3MC-treated cells (data not shown).

Finally, we studied the effect of MK571 on EROD activity to determine whether blockade of MRP1/MRP2 pumps could alter intracellular activity of arsenic. When Hep3B cells were incubated simultaneously with 3MC, As$_2$O$_3$, and MK571, inhibitory effects of As$_2$O$_3$ were significantly increased. In fact, 1 μM As$_2$O$_3$ was shown to decrease EROD activity to nearly 20% of that measured in Hep3B cells exposed to 3MC + MK571 (Fig. 8); in contrast, MK571 alone had no effect on EROD activity measured in 3MC-treated cells (data not shown).

**Discussion**

As$_2$O$_3$ has been recently shown to reduce growth of various solid and hematological tumor cells. In the present study, we demonstrated that this new anticancerous drug also down-regulates expression of human CYP1A1, which is involved in bioactivation of the chemical carcinogens PAHs, and in addition that its inhibitory effects are strongly increased by blockade of MRP1 and MRP2 transporter activities.

Our results showed that clinically relevant concentrations of As$_2$O$_3$, i.e., 0.25 to 5 μM (Shen et al., 1997), inhibited induction of EROD activity in both primary human hepatocytes (Fig. 5B). Taken together, these results suggested that As$_2$O$_3$ could act not only at transcriptional level but also at post-translational level.

Because major cellular effects of arsenic, including apoptosis, are mainly related to alterations of cellular redox status, involvement of ROS in As$_2$O$_3$-mediated inhibition of CYP1A1 was studied. Figure 6A shows that 5 μM As$_2$O$_3$ only slightly, although significantly, increased ROS production compared with 1 mM H$_2$O$_2$, used as a positive control. The effect of the potent reducing agent NAC on CYP1A1 activity was studied next. Hep3B cells were first pretreated with 20 mM NAC for at least 6 h and then cotreated with 3MC and As$_2$O$_3$ or 3MC and H$_2$O$_2$ along with 20 mM NAC. In these experimental conditions, NAC not only totally blocked ROS production in these cells but also restored maximal EROD activity (Fig. 6B). It is noteworthy that 1 mM H$_2$O$_2$ strongly reduced CYP1A1 activity in 3MC-treated Hep3B cells, which indicated that ROS production modulates CYP1A1 gene expression in these cells (Fig. 6B). However, other antioxidant agents, such as catalase, superoxide dismutase, nordihydroguaiaretic acid, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-noxyl, surprisingly, did not prevent As$_2$O$_3$-induced inhibition of EROD activity (data not shown), indicating that ROS production probably did not play a major role in cellular effects of As$_2$O$_3$ on CYP1A1 expression.

To further explain the mechanism of NAC effects, we determined whether this agent can interfere with arsenic transport in Hep3B cells. We first compared intracellular accumulation of arsenic in the absence or presence of NAC, as described above. Our results showed that arsenic amounts measured after a 3-h incubation with As$_2$O$_3$ were 34 ± 1.5 and 1.8 ± 0.3 ng/mg of protein in untreated and NAC-treated Hep3B cells, respectively (Fig. 7A). This marked alteration of cellular metalloid accumulation could result either from a reduced uptake or from an increased efflux of arsenic from Hep3B cells. To test the effect of NAC on efflux, cells were loaded with As$_2$O$_3$ in the absence of NAC and then incubated in arsenic-free medium in the presence of 20 mM NAC. As shown in Fig. 7B, NAC significantly accelerated arsenic efflux because the amounts of metalloid remaining, after a 3-h incubation in arsenic-free medium, were 10-fold lower in NAC-treated cells than those measured in untreated cells. We also examined the effects of MK571, a potent inhibitor of MRP1 and MRP2 efflux pumps, which are thought to mediate arsenic efflux from human cells (Kalå et al., 2000; Vernhet et al., 2001a). Figure 7B indicates that the remaining amounts of metalloid were significantly higher in cells treated with MK571 or MK571 and NAC than those measured in untreated cells or treated with NAC, respectively, thereby suggesting that blockade of a MK571-sensitive pump had lowered arsenic efflux. By Western blotting analysis, both MRP1 and MRP2 proteins were found to be highly expressed in Hep3B cells (data not shown). To ensure that NAC effect on efflux also mediated the decrease of metalloid intracellular accumulation, hepatoma cells were directly incubated simultaneously with 3MC, As$_2$O$_3$ and MK571 (Fig. 8); in contrast, MK571 alone had no effect on EROD activity measured in 3MC-treated cells (data not shown).

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Effects of As₂O₃ on CYP1A1 expression are consistent with those of arsenite in primary rat and human hepatocytes (Jacobs et al., 1999; Vakharia et al., 2001a) and HepG2 cells (Vakharia et al., 2001b) cotreated with various PAHs. However, it should be noted that arsenite was not found to prevent CYP1A1 induction in the mouse Hepa-1 (Maier et al., 2000, 2002) and the human lung adenocarcinoma CL3 (Ho and Lee, 2002) cell lines, suggesting that such effects of arsenic are complex and could be restricted to specific species and/or organs.

One major mechanism mediating cellular effects of therapeutic concentrations of As₂O₃ is enhancement of ROS production. Indeed, through inhibition of glutathione peroxidase, As₂O₃ has been shown to increase intracellular H₂O₂ levels and subsequently to induce selective caspase-dependent apoptosis of promyelocytic leukemia cells (Waxman and Anderson, 2001). Interestingly, H₂O₂ was recently reported to negatively regulate CYP1A1 expression in different cell lines by preventing activation of CYP1A1 promoter (Morel and Barouki, 1998). Although exogenous H₂O₂ could also down-regulate CYP1A1 expression in 3MC-treated Hep3B cells, this prooxidant agent did not seem to play a major role in inhibitory effect of As₂O₃. Indeed, H₂O₂ levels were only slightly increased in response to the highest concentration of metalloid, and, except NAC, no other antioxidant compound could reverse inhibition of EROD activity. It thus seems that transcriptional and post-translational effects of As₂O₃ were related to other mechanisms. It is well known that trivalent arsenic is a potent thioprine compound that interacts with critical cysteine residues of many intracellular proteins (Del Razo et al., 2001). For example, arsenite was recently demonstrated to prevent activation of the transcription factor nuclear factor-κB, by binding to the cysteine 179 in the activation loop of the IκB kinase catalytic subunit (Kapahi et al., 2000). It was also shown to down-regulate telomerase gene expression in human leukemia cells, partly through inhibition of DNA binding activity of Sp1, which contains redox-sensitive cysteine residues (Chou et al., 2001). It is noteworthy that Sp1 is a key regulatory trans-activating factor that binds to the basic transcription element on CYP1A1 promoter and increases induction of CYP1A1 gene expression by Ah receptor agonists (Kobayashi et al., 1996). Whether As₂O₃ may prevent 3MC-induced CYP1A1 expression in Hep3B cells by interfering with Sp1 DNA binding remains to be determined. Besides its effects on CYP1A1 expression, arsenic was shown to prevent, in rat hepatocytes, induction of CYP2B and CYP3A genes by phenobarbital and dexamethasone, respectively (Jacobs et al., 1999). Signaling pathways mediating up-regulations of CYP2B and CYP3A have been recently elucidated and mainly involve activation of the con-

![Fig. 8. Effects of MK571 on EROD activity in Hep3B cells.](image-url)
stittively active receptor and preganme X receptor, respectively (Sueyoshi and Negishi, 2001). These two transcription factors, which form dimers with the retinoid X receptor, have not been yet reported to be targeted by arsenic. On the other hand, activity of these mammalian P450s necessarily depends on the binding of iron heme to sulfur atom of a conserved cysteine residue in apoprotein (Gonzales, 1989). It could therefore be speculated that arsenic can block functional assembly of various P450s, including the human CYP1A1, by competing with heme for binding to the critical thiolate of apoprotein. Alternatively, arsenic may have reduced cellular heme pool because it is known to strongly increase heme oxygenase activity in different cell lines. However, pretreatment of Hep3B cells with heme was not found to reduce inhibitory effects of the metalloid on CYP1A1 activity, which thus ruled out this hypothesis (L. Vernhet, M. Le Vee, and O. Fardel, unpublished data). Further studies are required to clarify differences between protein levels and corresponding enzyme activities.

The present study also demonstrated that NAC prevented As$_2$O$_3$-induced inhibition of EROD activity by strongly reducing intracellular accumulation of the metalloid. The mechanism of NAC is complex and involves a dual effect on arsenic transport. When incubated with As$_2$O$_3$-loading cells, NAC was found to markedly stimulate arsenic efflux, likely through MRPl and/or MRPl2 pumps, because MK571 totally blocked its effect. It was recently shown that MRPl can transport arsenic as a complex with glutathione (Kala et al., 2000), and we previously reported that MRPl-mediated arsenic efflux from As$_2$O$_3$-loaded cells was strongly dependent on glutathione levels (Vernhet et al., 2001a). Thus, accumulation of large amounts of NAC into Hep3B cells could have increased the formation of suitable arsenic-containing complexes for MRPl- or MRPl2-mediated metalloid transport. On the other hand, when directly coexposed with As$_2$O$_3$, NAC seems to mainly inhibit arsenic uptake. Indeed, arsenic retention in NAC-treated cells was only weakly increased by MK571 compared with that of cells not exposed to NAC. NAC was also found to reduce As$_2$O$_3$ uptake in HepG2 cells, suggesting that this effect was not restricted to a particular cell type. This result is important because many studies have reported a protective role of NAC when added simultaneously with arsenic (Dai et al., 1999; Lu et al., 2000; Duymdam et al., 2001). In such studies, NAC seemed to block cellular effects of arsenic through antioxidant properties or direct intracellular interaction with the metalloid. Pathways of arsenic uptake in human cells are largely unknown and, thus, mechanisms of NAC effects remain to be precise. However, it is noteworthy that similarly NAC blocks cellular uptake of cadmium, another thioloprine compound (Wisniewo et al., 1998). Blockade of MRPl/MRPl2-mediated arsenic efflux by MK571 was also associated with a significant enhancement of As$_2$O$_3$ inhibitory effects on 3MC-induced CYP1A1 activity. These results are consistent with our previous observations showing that increased levels of MRPl expression prevent As$_2$O$_3$ toxicity in human tumor cells and further strengthen the role of such pumps in controlling intracellular active pool of arsenic. More generally, this suggests that cellular activity of transporters, such as MRPl and MRPl2, represents a critical parameter influencing the effects of compounds interacting with drug-metabolizing enzymes. In this context, P-glycoprotein-mediated transport of rifam-


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