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Disulfiram Metabolites Permanently Inactivate the Human Multidrug Resistance P-Glycoprotein†

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Abstract: The human multidrug resistance P-glycoprotein (P-gp) uses ATP to transport a wide variety of structurally unrelated cytotoxic compounds out of the cell. The relatively high expression of P-gp in organs such as the intestine, kidney, blood−brain/testes barrier and in some tumor cells can compromise chemotherapy treatments for patients with cancer or AIDS/HIV. It has been difficult to inhibit P-gp during chemotherapy with noncovalent inhibitors because the relatively high levels of inhibitors have severe side effects. An alternative approach to inhibit P-gp would be to covalently modify cysteine residues within the NBDs. In this study, we tested whether metabolites of disulfiram, a drug currently used to treat chronic alcoholism, could inhibit P-gp. We show that the disulfiram metabolites, *S*-methyl *N,N*-diethylthiocarbamate sulfoxide and *S*-methyl *N,N*-diethylthiocarbamate sulfone inhibited the verapamil-stimulated ATPase activity of P-gp with IC_{50} values (concentrations that result in 50% inhibition of activity) of 9 and 4.8 μM, respectively. Similarly, *S*-methyl *N,N*-diethylthiocarbamate sulfoxide and *S*-methyl *N,N*-diethylthiocarbamate sulfone inhibited the activity of aldehyde dehydrogenase with IC_{50} values of 3.2 and 1.7 μM, respectively. Inhibition of P-gp by the metabolites was not reversed by addition of the reducing compound, dithiothreitol. We then determined which endogenous cysteine residue was responsible for inhibiting P-gp activity after exposure to the disulfiram metabolites. Treatment of P-gp mutants containing a single cysteine residue showed that inactivation was primarily due to modification of Cys1074 in NBD2. These results indicate that metabolites of disulfiram can covalently inactivate P-gp. Covalent modification of drug transporters could be a useful approach for inhibiting their activities during chemotherapy.

Keywords: P-glycoprotein; aldehyde dehydrogenase; disulfiram; disulfiram metabolites; ATPase activity; covalent inhibition

Introduction

Chemotherapy is the most effective treatment for metastatic tumors and hematological malignancies. It is the treatment of choice for patients who have cancers that do not respond to surgery or radiation (about 50% of total cancer cases). Unfortunately, cancer cells can develop resistance to chemotherapeutic agents. A particular problem is the development of multidrug resistance whereby cells selected for resistance to a single drug also show cross-resistance to other structurally diverse compounds with different intracellular targets. Chemotherapy would be a more effective treatment for cancer if the problem of drug resistance could be overcome.

The first important protein implicated in the phenomenon of multidrug resistance was P-gp,[1] the product of the MDR1 (or ABCB1) gene.[2,3] P-gp is an ATP-dependent drug efflux

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pump with a very broad drug specificity.[4] It confers resistance by pumping drugs out of the cell. P-gp is a member of the ATP-binding cassette (ABC) family of transporters that consists of 48 human members.[5] While there are many causes of multidrug resistance,[6] there is ample evidence that inhibition of P-gp would improve cancer chemotherapy.

Inhibitors of P-gp have been employed to try to improve the effectiveness of chemotherapy. Inactivation of P-gp should have no ill effects on patients, at least in the short term, because studies on knockout mice show that the protein is not essential.[7] Very encouraging results were achieved in retinoblastoma patients with the P-gp inhibitor cyclosporin A. Use of short high-dose cyclosporin infusions at the same time as vincristine achieved a high cure rate.[8,9]

There are limitations, however, in the P-gp inhibitors developed to date. One problem is that many of the early agents that reversed multidrug resistance were used at concentrations that caused unacceptable toxicity.[10,11] Another problem is that most inhibitors act by interfering with drug binding. Potential problems are that (1) inhibition is reversible; (2) inhibition may not be effective with a high-affinity anticancer drug; (3) P-gp contains multiple binding sites[12] so only a subset of transport sites may be blocked; (4) substrate-induced conformational changes could reduce the affinity of the inhibitor;[13] and (5) P-gp modulators can induce expression of the protein[6,14] and enhance its maturation and delivery to the cell surface.[15]

An alternative approach would be to irreversibly inhibit P-gp by covalently modifying essential cysteines[16] located in the Walker A sites in the NBDs using disulfiram metabolites. Disulfiram is an interesting compound because it has been used for over 50 years to treat chronic alcoholism.[17] It inactivates aldehyde dehydrogenase (ADH) by modification of an essential cysteine residue.[18,19]

We previously showed that disulfiram could inhibit P-gp ATPase activity in vitro by reacting with cysteines in the Walker A consensus sites for nucleotide binding.[20] Disulfiram reaction with P-gp in vivo, however, would be rapidly reversed because of reduction of the disulfide bond by glutathione in the cells. A similar situation exists for ADH. The enzyme is inhibited by treatment with disulfiram, in vitro but the inhibition is readily reversed or prevented by cellular glutathione. The reason that disulfiram can inhibit ADH when

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administered to patients is that disulfiram is converted to metabolites that can irreversibly react with thiol groups. Disulfiram is rapidly metabolized in vivo to *N*,*N*-diethylthiocarbamate and ultimately to the proposed active metabolites of disulfiram: *S*-methyl *N*,*N*-diethylthiocarbamate sulfoxide (DETC-sulfoxide) and *S*-methyl *N*,*N*-diethylthiocarbamate sulfone (DETC-sulfone).[21−23]

In this study we tested whether P-gp could be permanently inactivated by reaction with metabolites of disulfiram.

Experimental Section

Construction of Mutants. Wild-type P-gp cDNA was modified to encode for a $(His)_{10}$ tag to facilitate purification of the protein by nickel-chelate chromatography.[24] The construction of an active Cys-less P-gp was done by replacing the seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227 with alanines.[25] P-gp mutants containing only one endogenous cysteine were made as described previously.[16]

Expression of P-gp Mutants and Measurement of Verapamil-Stimulated ATPase Activities. To measure P-gp ATPase activity in membranes, wild-type or mutant P-gp cDNAs were introduced into a baculovirus vector and recombinant virus was used to infect insect Sf9 cells as described previously.[16] Membranes from Sf9 cells infected with recombinant or control baculovirus were prepared[16] and suspended in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5 or 8.8) at a protein concentration of 1−2 mg/mL. To test for inhibition by metabolites of disulfiram, the membranes were incubated with various concentrations of disulfiram, DETC-sulfoxide, or DETC-sulfone (synthesized by Toronto Research Chemicals, Toronto, Ontario) for 30 min at 20 °C. Stocks of the disulfiram metabolites were prepared in methanol. Verapamil-stimulated ATPase activity was then determined by addition of an equal volume of buffer containing 200 mM Tris-HCl, pH 7.5, 150 mM NaCl, 8 mM EGTA, 4 mM ouabain, 20 mM sodium azide, 8 mM DTT, 20 mM $MgCl_2$, 10 mM ATP and with or without 80 μM verapamil. Maximal drug-stimulated ATPase activity of P-gp in Sf9 membranes is observed in the presence of 40 μM verapamil. The amount of inorganic phosphate liberated was determined by the method of Chifflet et al.[26]

To determine whether metabolites of disulfiram were substrates of P-gp, the mutant P-gps were isolated by nickel-chelate chromatography, mixed with lipid, and assayed for drug-stimulated ATPase activity. Fifty 10 cm diameter plates of HEK 293 cells were transfected with histidine-tagged wild-type or mutant P-gp cDNAs. After 24 h, the medium was replaced with fresh medium containing 10 μM cyclosporin A. Cyclosporin A is a substrate of P-gp and acts as a powerful specific chemical chaperone to enhance the maturation and yield of the mutant P-gps.[15,27] The cells were then harvested 24 h later and P-gp$(His)_{10}$ was isolated by nickel-chelate chromatography as described previously.[24] The isolated P-gps were mixed with an equal volume of 10 mg/mL crude sheep brain phosphatidylethanolamine (type II-S, Sigma) and sonicated. The samples were treated with disulfiram or its metabolites and then assayed for verapamil-stimulated ATPase activity by addition of an equal volume of ATPase buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM ATP, and either no drug or 2 mM verapamil. This concentration of verapamil caused maximal stimulation of the ATPase activity of Cys-less P-gp when reconstituted with lipid. The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate liberated was determined.[26]

Aldehyde Dehydrogenase Assay. Yeast ADH activity was assayed by measuring the production of NADH during oxidation of acetaldehyde.[28] ADH from bakers' yeast (Sigma-Aldrich) was suspended in 0.1 M pyrophosphate buffer, pH 8.8 (20 units/mL) and desalted by being passed twice through gel filtration columns (CENTRI.SPIN-20 columns, Princeton Separations) that were equilibrated with 0.1 M pyrophosphate buffer, pH 8.8. After treatment with or without various concentrations of DETC-sulfoxide or DETC-sulfone, the activity was measured by diluting the enzyme to 0.2 unit/mL in a reaction mixture consisting of 65 mM pyrophosphate, pH 8.8, 25 mM KCl, 500 μM NAD, and 125 μM acetaldehyde. The mixture was incubated at 25 °C for 15 min and cooled on ice, and the amount of NADH produced was determined by measurement of the absorbance at 340 nm.

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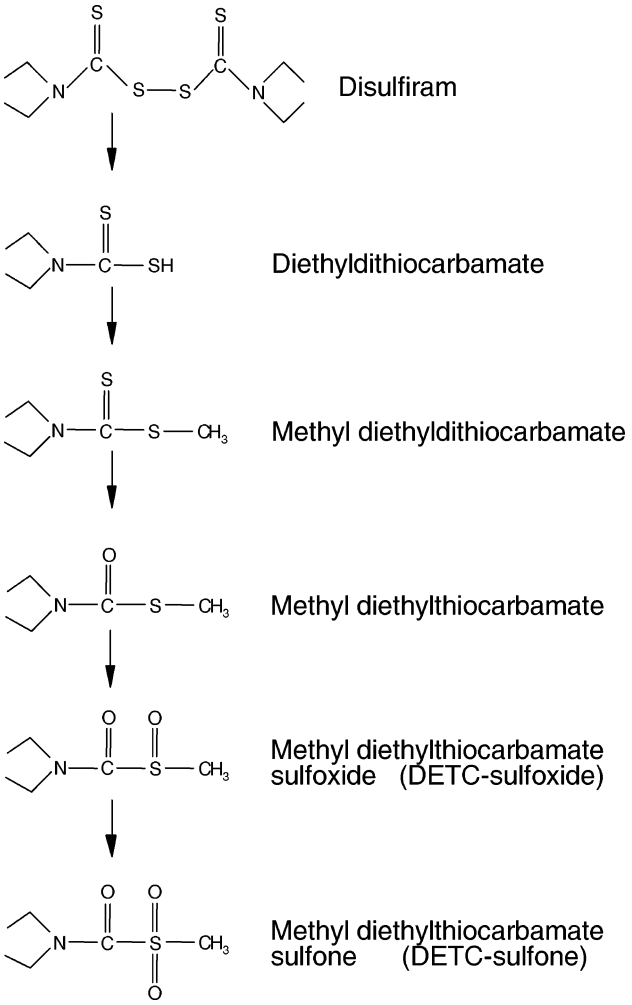

Figure 1. Metabolism of disulfiram. The predicted metabolic pathway of disulfiram is shown.[31,51] Disulfiram is reduced in the bloodstream to diethyldithiocarbamate. Diethyldithiocarbamate is then methylated by thiol methyltransferase or thiopurine methyltransferase to yield methyl diethyldithiocarbamate, which is then oxidized by cytochrome P450s to give methyl diethylthiocarbamate. Further oxidation by cytochrome P450s would yield methyl diethylthiocarbamate sulfoxide (DETC-sulfoxide) and methyl diethylthiocarbamate sulfone (DETC-sulfone).

Results

Disulfiram is used in aversion therapy for chronic alcoholism because it causes inhibition of ADH activity.[18] Inhibition of ADH in vivo likely involves a metabolite of disulfiram because maximal inhibition in rats occurs 8 h after administration.[29] In addition, treatment of rats with cytochrome P450 inhibitors prior to disulfiram administration blocks inhibition of ADHs.[30] Disulfiram is rapidly metabolized following administration, and the important metabolites that inhibit ADH have been identified (Figure 1).[31]

The clinically important metabolites of disulfiram are *S*-methyl *N*,*N*-diethylthiocarbamate sulfoxide (DETC-sulfoxide) and *S*-methyl *N*,*N*-diethylthiocarbamate sulfone (DETC-sulfone) (Figure 1). The DETC-sulfoxide and DETC-sulfone metabolites of disulfiram appear to be responsible for in vivo inhibition of ADH.[32−34] These metabolites inhibit ADH by carbamoylating a critical cysteine (Cys302 in rat mitochondrial ADH).[19,35]

Wild-type P-gp has seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227. Therefore, we determined whether DETC-sulfoxide or DETC-sulfone could inhibit the activity of P-gp through reaction with endogenous cysteines. Relatively high levels of P-gp can be obtained by expressing recombinant baculovirus containing P-gp cDNA in insect Sf9 cells.[36] The membranes prepared from infected Sf9 cells contain enough P-gp that drug-stimulated ATPase activity can be determined without purification of P-gp. Accordingly, membranes were prepared from Sf9 insect cells infected with recombinant wild-type or Cys-less P-gp. We previously showed that Cys-less P-gp in which all seven endogenous cysteines were mutated to alanines was still active.[25] The membranes were then treated with various concentrations (0−200 μM) of DETC-sulfoxide or DETC-sulfone for 30 min at 20 °C and then assayed for verapamil-stimulated ATPase activity. Verapamil-stimulated ATPase activity was measured because drug binding to P-gp is coupled to ATPase activity,[37] and there is a good correlation between ATPase activity and turnover of drug

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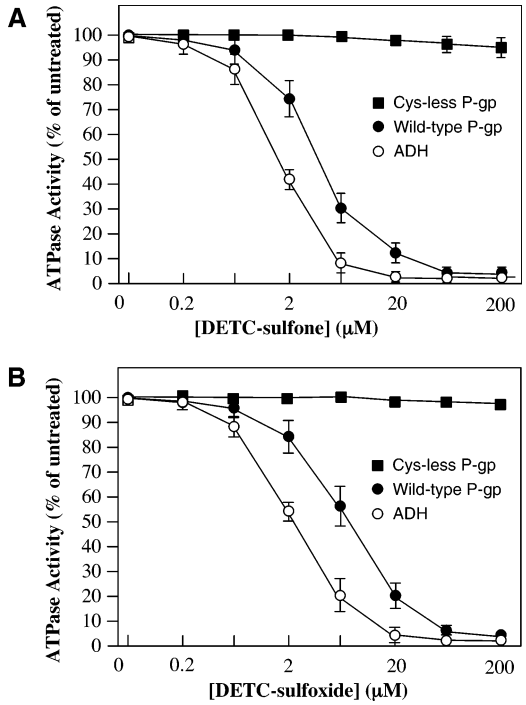

Figure 2. Inhibition of wild-type and Cys-less P-gp and ADH by DETC-sulfoxide or DETC-sulfone. ADH or membranes prepared from Sf9 cells expressing wild-type or Cys-less P-gp were incubated for 30 min at 20 °C in the presence or absence of various concentrations of DETC-sulfone (A) or DETC-sulfoxide (B). The activities of the ADH samples were determined by measuring the production of NADH during oxidation of acetaldehyde. The P-gp activities were determined by assaying for verapamil stimulation of ATPase activity. The activities are expressed relative to the sample that was not treated with DETC-sulfone or DETC-sulfoxide. Each value is the average of triplicate measurements.

substrates during P-gp transport.[38] Yeast ADH (Sigma-Aldrich) was also treated with DETC-sulfoxide or DETC-sulfone for 30 min at 20 °C and served as controls for determining whether the conditions used were optimal for inhibition of activity. The activity of yeast ADH was determined by measuring the reduction of NAD^+ during oxidation of acetaldehyde to acetic acid.[28] Figure 2A shows the results obtained with DETC-sulfone. DETC-sulfone inhibited the activity of ADH with an IC_{50} of 1.7 μM and indicated that the conditions chosen were optimal. The ATPase activity of wild-type P-gp was also inhibited by DETC-sulfone (IC_{50} of 4.8 μM). By contrast, the activity of Cys-less P-gp was not inhibited by DETC-sulfone since the enzyme retained more than 95% of its activity even after exposure to 200 μM DETC-sulfone. Similar results were obtained with DETC-sulfoxide (Figure 2B). The activities of wild-type P-gp and ADH were inhibited by DETC-sulfoxide with IC_{50} values of 9.0 and 3.2 μM, respectively. DETC-sulfoxide did not affect the activity of Cys-less P-gp because more than 95% of the ATPase activity was retained after exposure to 200 μM DETC-sulfoxide (Figure 2B).

Disulfiram can inhibit P-gp or ADH by reacting with individual cysteine residues or by cross-linking endogenous cysteines.[20,35,39−41] Therefore, we tested whether DETC-sulfoxide or DETC-sulfone could cross-link P-gp. Cross-linking between cysteine residues in P-gp is readily detected because the cross-linked protein migrates with lower mobility than the 170 kDa mature P-gp in SDS−PAGE gels.[42,43] To obtain relatively high levels of mature 170 kDa P-gp, expression was carried out in HEK 293 cells. Membranes were prepared from HEK 293 cells expressing wild-type or Cys-less P-gp and treated for 10 min at 37 °C with (0.1 or 1 mM) disulfiram. The samples were solubilized with SDS sample buffer and subjected to immunoblot analysis. Figure 3A shows that treatment of wild-type P-gp with disulfiram resulted in a band that migrated with lower electrophoretic mobility than the 170 kDa P-gp. This cross-linked product was not detected when Cys-less P-gp was treated with disulfiram (Figure 3B). These results are consistent with those reported by Sauna et al.[39] Membranes containing wild-type P-gp were then treated with (0.1 or 1 mM) DETC-sulfoxide or DETC-sulfone. Figure 3C,D shows that cross-linked product was not detected when wild-type P-gp was treated with DETC-sulfoxide or DETC-sulfone. These results show that DETC-sulfoxide and DETC-sulfone likely inhibit P-gp by covalently modifying one or more endogenous cysteine residues and not by cross-linking of endogenous cysteine residues.

We then determined whether inhibition of wild-type P-gp by DETC-sulfoxide and DETC-sulfone was through modification of one or more endogenous cysteine residues. Accordingly, histidine-tagged wild-type P-gp, Cys-less P-gp, and Cys-less P-gp containing a single endogenous cysteine (positions 137, 431, 717, 956, 1074, 1125, or 1227) were

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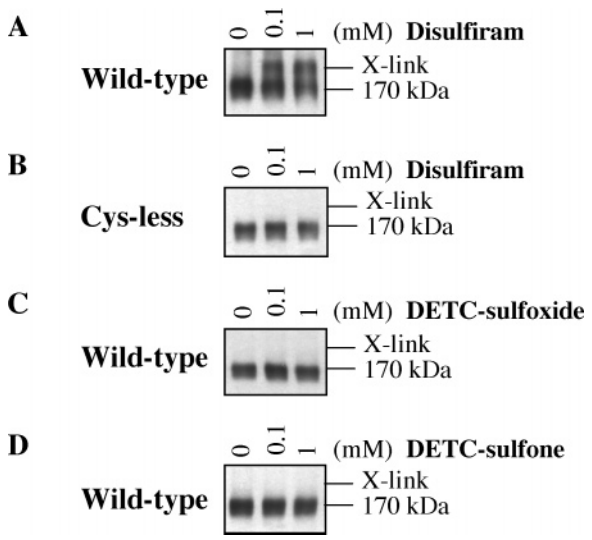

Figure 3. Disulfide cross-linking of P-gp. Membranes prepared from HEK 293 cells expressing wild-type or Cys-less P-gp were treated with 0, 0.1, or 1 mM disulfiram, DETC-sulfoxide, or DETC-sulfone for 10 min at 37 °C. Samples were solubilized with SDS sample buffer and then subjected to immunoblot analysis with rabbit polyclonal antibodies against P-gp[52] followed by enhanced chemiluminescence (Pierce, Rockford, IL.). The positions of mature (170 kDa) and cross-linked (X-link) P-gp are indicated.

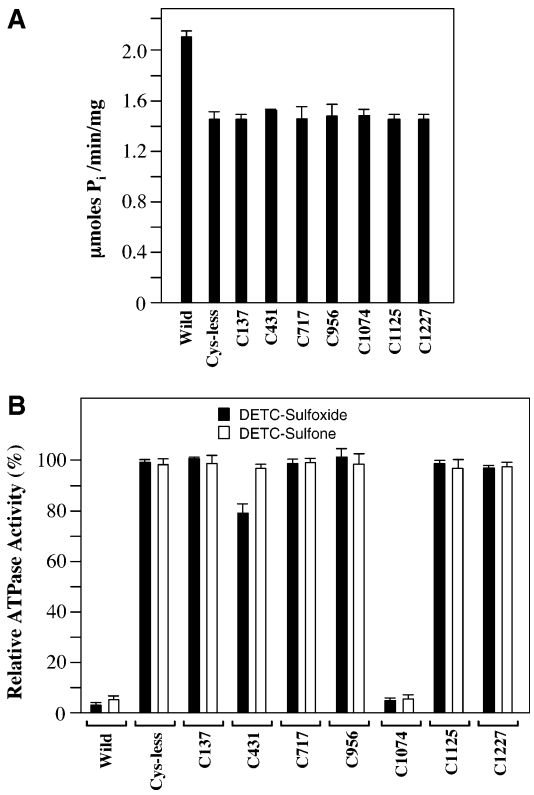

Figure 4. Inhibition of P-gp cysteine mutants. Histidine-tagged wild-type P-gp, Cys-less P-gp, or Cys-less P-gp mutants containing an endogenous cysteine at positions 137, 431, 717, 956, 1074, 1125, or 1227 were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. (A) The isolated P-gps were mixed with lipid and assayed for verapamil-stimulated ATPase activity. (B) Samples were then treated with 200 μM DETC-sulfoxide, 200 μM DETC-sulfone, or no metabolites for 30 min at 20 °C. The samples were then assayed for verapamil-stimulated ATPase activity. Each value is the average of triplicate assays. The ATPase activity (%) is expressed relative to that of a sample not treated with DETC-sulfoxide or DETC-sulfone.

expressed in HEK 293 cells, isolated by nickel chromatography, and assayed for verapamil-stimulated ATPase activity in the presence of lipid. In agreement with earlier studies,[16,25] we found that Cys-less P-gp and the single cysteine mutants retained about 70% of the verapamil-stimulated ATPase activity of wild-type enzyme (Figure 4A). The isolated P-gps were then treated with 200 μM DETC-sulfoxide or DETC-sulfone and then assayed for verapamil-stimulated ATPase activity. Figure 4B shows the effect of DETC-sulfoxide or DETC-sulfone on the verapamil-stimulated ATPase activity of wild-type P-gp, Cys-less P-gp, and the single cysteine mutants (Figure 4B) relative to a sample that was not treated with the disulfiram metabolite. The ATPase activity of wild-type P-gp was inhibited by more than 90% by DETC-sulfoxide or DETC-sulfone, while that of Cys-less P-gp was unaffected by these metabolites. The activity of Cys-less P-gp mutant containing endogenous cysteine C1074 in NBD2 was particularly sensitive to inhibition by both DETC-sulfoxide and DETC-sulfone (Figure 4B). By contrast, the activity of P-gp mutant C431 (in NBD1) was not inhibited by DETC-sulfone and was inhibited only slightly (about 20%) by DETC-sulfoxide. The activities of other Cys-less P-gp mutants with endogenous cysteines at positions 137, 717, 956, 1125, or 1227 were not inhibited by DETC-sulfoxide or DETC-sulfone (Figure 4B).

We then tested whether inhibition of wild-type P-gp activity by disulfiram, DETC-sulfoxide, or DETC-sulfone could be reversed with the reducing agent, dithiothreitol (DTT). Histidine-tagged wild-type P-gp was treated with disulfiram, DETC-sulfoxide, or DETC-sulfone followed by exposure to 20 mM DTT at pH 7.5 or 8.8. The samples were treated at these pHs because inhibition of ADH by DETC-sulfone has been reported to be reversible by DTT at pH 8.8 but not at pH 7.5.[40] The ADH and wild-type P-gp activities were strongly inhibited by disulfiram, DETC-sulfoxide, or DETC-sulfone (Figure 5A). The samples treated with disulfiram, DETC-sulfoxide, or DETC-sulfone were then treated with DTT at pH 7.5 or pH 8.8, and activity was determined. Figure 5B shows that the activity of both ADH and P-gp after treatment with disulfiram could be reversed at pH 7.5 or 8.8. The inhibitory effects of DETC-sulfoxide or DETC-sulfone on ADH activity could be reversed when the samples were exposed to DTT at pH 8.8 but not at pH 7.5. By contrast, inhibition of P-gp activity by DETC-sulfoxide or DETC-sulfone could not be reversed by DTT at either pH.

Inhibition of P-gp by DETC-sulfoxide or DETC-sulfone differs from that by disulfiram because modification of the

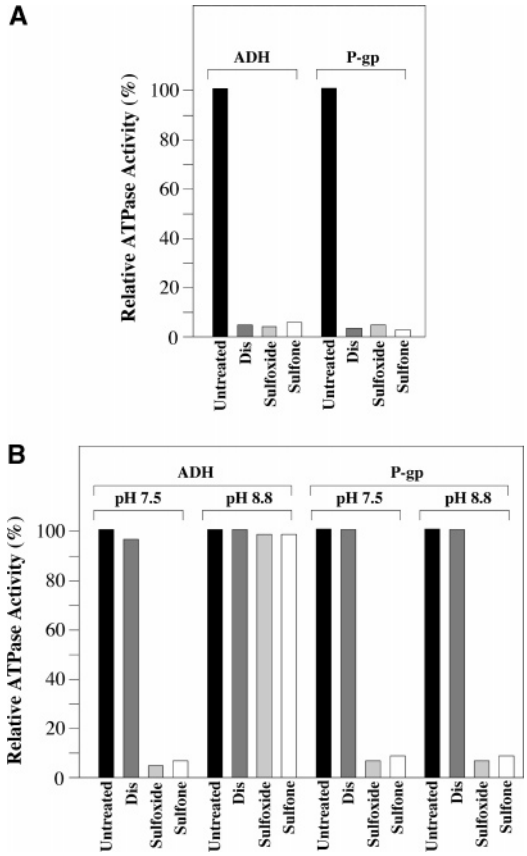

Figure 5. Effect of DTT and pH on the activity of P-gp or ADH treated with disulfiram or its metabolites. (A) Isolated histidine-tagged wild-type P-gp was mixed with lipid and sonicated. Both P-gp and ADH were then incubated with no additions (Untreated) or with 200 μM disulfiram (Dis), DETC-sulfoxide (Sulfoxide), or DETC-sulfone (Sulfone) for 30 min at 20 °C in TBS pH 8.8. The ADH activity and verapamil-stimulated P-gp ATPase activities of the treated samples were determined. (B) The P-gp and ADH samples treated with no additions (Untreated) or with 200 μM disulfiram (Dis), DETC-sulfoxide (Sulfoxide), or DETC-sulfone (Sulfone) were then diluted 10-fold into 100 mM Tris-HCl, pH 7.5 or 8.8 containing 20 mM DTT. After 10 min at 20 °C, the activities of the enzymes were measured. Each value is the average of duplicate assays and is expressed relative to that of an untreated sample.

cysteine residues was not reversed by DTT. Another difference between inhibition by the metabolites DETC sulfoxide/sulfone and by disulfiram is that both metabolites (0−1 mM) did not stimulate the ATPase activity or inhibit the verapamil-stimulated ATPase activity of Cys-less P-gp (data not shown). By contrast, disulfiram stimulated (8-fold) the ATPase activity of Cys-less P-gp.[20] These results show that DETC-sulfoxide and DETC-sulfone are not substrates of P-gp.

Discussion

Disulfiram is a particularly interesting inhibitor of P-gp because of the numerous ways it can inhibit the protein's ability to confer drug resistance. First, the presence of disulfiram during synthesis of P-gp inhibits maturation of the protein.[20] Second, disulfiram is a substrate of P-gp so it can act as a competitive inhibitor for drug binding and transport.[20,39] Finally, disulfiram can inhibit P-gp by directly reacting with either cysteine within the Walker A sites for ATP-binding or promoting cross-linking between these two cysteines.[20,39] Unfortunately, these types of inhibition can be readily reversed by dilution of the enzyme or incubation in the presence of thiol reducing agents such as glutathione. Indeed, permanent inactivation of wild-type P-gp was not observed when it was isolated from NIH 3T3 or HEK 293 cells that had been treated with disulfiram for 24 h (unpublished observations). Therefore, it is unlikely that inhibition by disulfiram reported in earlier in vivo studies with HEK 293[20] or NIH 3T3 cells[39] was due to reaction with disulfiram metabolites because neither of these cell lines expresses the required cytochrome P450s needed to convert disulfiram to its metabolites.[44,45] Inhibition of P-gp by the disulfiram metabolites DETC-sulfoxide or DETC-sulfone reported in this study, however, has the advantage of being irreversible under normal cellular conditions.

Inactivation of ADH during aversion therapy in chronic alcoholism likely involves carbamoylation of an essential cysteine. Treatment of rat mitochondrial ADH in vitro with DETC-sulfoxide followed by proteolysis and mass spectrometry showed that inhibition of activity was due to carbamoylation of Cys302.[35,46] Similar findings were reported when mitochondrial ADH was isolated from rats that had been administered disulfiram.[19,47] Like ADH, we found that inhibition of P-gp with either DETC-sulfoxide or DETC-sulfone was mainly due to modification of one particular cysteine, Cys1074 in NBD2 (Figure 6). It has been shown that modification of only one of the Walker A cysteines with *N*-ethylmaleimide[16,48] or with disulfiram[20] was sufficient to inactivate P-gp. A difference between carbamoylation of Cys302 in rat ADH and Cys1074 of P-gp was that the reaction was not reversible when modified P-gp was incubated with DTT at pH 8.8. It has been shown that carbamoylation of rat ADH can be reversed when the

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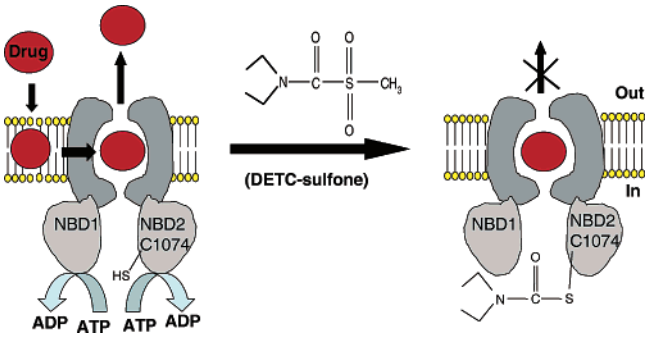

Figure 6. Model of inhibition of P-gp by DETC-sulfone. P-gp functions as a drug pump to extract drug substrates out of the membrane and then transports the drugs back out of the cell. Transport requires ATP hydrolysis at both NBDs.[16] Exposure of P-gp to the disulfiram metabolite DETC-sulfone causes permanent inactivation through carbamoylation of Cys1074 in NBD2. Cys1074 is depicted as being more reactive with DETC-sulfone than Cys431. Drug substrates are likely to still bind to P-gp because the NBDs are not required for drug binding. A P-gp deletion mutant lacking both NBDs can still bind drug substrate.[53] Drug transport is blocked when modification of only one cysteine in a Walker A site prevents ATP hydrolysis at both sites.

modified enzyme was incubated in the presence of DTT at pH 8.8.[40] The product of carbamoylation of P-gp, however, resembles the stable carbamoyl adduct formed with Cys125 of rat hemoglobin β-chain after treatment with DETC-sulfoxide.[31] It was found that carbamoylation of rat hemoglobin could not be reversed by DTT at pH 8.5 or 10.5 at 37 °C or 56 °C.

An interesting observation was that Cys1074 (in NBD2) was more reactive than C431 (in NBD1) to DETC-sulfoxide or DETC-sulfone even though both cysteines are located at equivalent positions within the Walker A sequences. Cysteine 1074 has also been reported to be more sensitive to modification by other thiol-reactive compounds such as *N*-ethylmaleimide[16] and disulfiram.[20] Cys1074 is more reactive than Cys431 because it may be more accessible to the thiol-reactive compounds or the pK_a values of the thiol groups are different. In addition, the Cys431 in the Walker A sequences in NBD1 (GNSGCGKS) and Cys1074 in NBD2 (GSSGCGKS) have slightly different neighboring residues, and this may be related to the fact that the two ATP-binding sites are not symmetric.[16,49,50]

The results of this study show that both metabolites of disulfiram (DETC-sulfoxide or DETC-sulfone) can permanently inactivate P-gp. Therefore, development of covalent inhibitors could represent a novel method for inactivating drug transporters.

Abbreviations Used

P-gp, P-glycoprotein; ADH, aldehyde dehydrogenase; ABC, ATP-binding cassette; DETC-sulfoxide, *S*-methyl *N*,*N*-diethylthiocarbamate sulfoxide; DETC-sulfone, *S*-methyl *N*,*N*-diethylthiocarbamate sulfone; DTT, dithiothreitol; HEK, human embryonic kidney; NBD1, first nucleotide-binding domain; NBD2, second nucleotide-binding domain; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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