0026-895X/04/6503-675–684
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:675–684, 2004

Vol. 65, No. 3
2886/1129866
Printed in U.S.A.

The Molecular Basis of the Action of Disulfiram as a Modulator of the Multidrug Resistance-Linked ATP Binding Cassette Transporters MDR1 (ABCB1) and MRP1 (ABCC1)

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Received August 6, 2003; accepted October 21, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The overexpression of multidrug resistance protein 1 (MDR1) and multidrug resistance protein 1 (MRP1) gene products is a major cause of multidrug resistance in cancer cells. A recent study suggested that disulfiram, a drug used to treat alcoholism, might act as a modulator of P-glycoprotein. In this study, we investigated the molecular and chemical basis of disulfiram as a multidrug resistance modulator. We demonstrate that in intact cells, disulfiram reverses either MDR1- or MRP1-mediated efflux of fluorescent drug substrates. Disulfiram inhibits ATP hydrolysis and the binding of [α-^{32}P]8-azidoATP to P-glycoprotein and MRP1, with inhibition curves comparable with those of *N*-ethylmaleimide, a cysteine-modifying agent. However, if the ATP sites are protected with excess ATP, disulfiram stimulates ATP hydrolysis by both transporters in a concentration-dependent manner. Thus, in addition to modifying cysteines at the ATP sites, disulfiram may interact with the drug-substrate binding site. We demonstrate that disulfiram, but not *N*-ethylmaleimide, inhibits in a concentration-dependent manner the photoaffinity labeling of the multidrug transporter with ^{125}I-iodoarylazidoprazosin and [^{3}H]azidopine. This suggests that the interaction of disulfiram with the drug-binding site is independent of its role as a cysteine-modifying agent. Finally, we have exploited MRP4 (ABCC4) to demonstrate that disulfiram can inhibit ATP binding by forming disulfide bonds between cysteines located in the vicinity of, although not in, the active site. Taken together, our results suggest that disulfiram has unique molecular interactions with both the ATP and/or drug-substrate binding sites of multiple ATP binding cassette transporters, which are associated with drug resistance, and it is potentially an attractive agent to combat multidrug resistance.

Cells resistant to chemically diverse drugs with multiple mechanisms of action are defined as exhibiting the multidrug resistance (MDR) phenotypes. In most cases, decreased influx and/or increased efflux of drugs from cells, resulting in decreased accumulation of drugs in cells, is responsible for MDR (Gottesman et al., 2002). The overexpression of the energy-dependent efflux membrane proteins P-glycoprotein (Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1), and the mitoxantrone-resistance–associated protein (ABCG2) have been strongly associated with MDR (Gottesman et al., 2002). These are all members of the ATP-binding cassette (ABC) family of proteins (Borst and Elferink, 2002). Because there is sufficient evidence to implicate several ABC transport proteins as negative prognostic markers during cancer chemotherapy (Gottesman, 2002), the pharmacological reversal of Pgp and, to a limited extent, MRP1 function has been a major focus of research (Tan et al., 2000). However, although it was demonstrated as early as 1982 that verapamil augments the antiproliferative effect of vincristine, success in overcoming MDR has been limited (Tan et al., 2000; Gottesman et al., 2002).

A novel strategy to combat MDR has recently been propounded that involves the use of disulfiram (Antabuse; Odyssey Pharmaceuticals, East Hanover, NJ), a drug approved for use in treating alcoholism (Loo and Clarke, 2000a). The failure of several first-generation MDR modulators in clinical trials has led to the view that such drugs, originally developed for other targets, may not be useful in combating MDR (Leonard et al., 2002). The most significant drawback of such compounds is that they inhibit MDR pumps at relatively high concentrations. Attempts to achieve these concentrations in vivo result in severe adverse events and toxicity. This is

ABBREVIATIONS: MDR, multidrug resistance; ABC, ATP binding cassette; Pgp, P-glycoprotein; IAAP, [^{125}I]iodoarylazidoprazosin; MRP, multidrug resistance protein; HEK, human embryonic kidney; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; Vi, sodium orthovanadate; P_i, inorganic phosphate; MK571, 3-([[3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl)-((3-dimethylamino-3- oxopropyl)-thio)-methyl]thio)propanoic acid; bodipy-FL, 4,4-difluoro-5,7-dimethyl-4-bora-*3a*,*4a*-diaza-*s*-indacene-3-pentanoic acid, succinimidyl ester.

unlikely to be a significant issue with disulfiram (Chick, 1999). The LD_{50} values in rats for cyclosporin A and verapamil are 25 mg/kg and 16 mg/kg, respectively, whereas that for disulfiram is 8.6 g/kg. The normal adult dose of disulfiram in the treatment of alcoholism, for example, is 500 mg/day, and more than five decades of clinical experience has shown that this dose can be safe over extended periods. It has been estimated that the current clinical dose of disulfiram translates to an approximate concentration of 20 μM in a 100-kg subject (Loo and Clarke, 2000a). These levels are likely to be sufficient to achieve the biochemical effects described in this work. Moreover, the toxicity data suggest that if necessary, the dosage can be increased substantially while keeping adverse events manageable. In this work, therefore, we have investigated in detail the biochemical basis of disulfiram as an MDR modulator.

The previous work investigating the effect of disulfiram on Pgp showed that disulfiram can reduce Pgp-mediated drug resistance by inhibiting the maturation of Pgp. It was suggested that disulfiram might also interact with Pgp, inhibiting the substrate-stimulated ATP hydrolysis (Loo and Clarke, 2000a). The work reported here demonstrates that disulfiram reverses both Pgp and MRP1-mediated efflux in intact cells, and consistent with its role as a cysteine-modifying agent, disulfiram inhibits [α-^{32}P]8-azidoATP binding and ATP hydrolysis by Pgp and MRP1. Interestingly, however, we present several lines of evidence that the interactions between disulfiram and Pgp inhibit the binding of drug substrates. Finally, we show that disulfiram forms intramolecular disulfide bonds and can obstruct the ATP-binding pocket not only by covalently binding to cysteines in the active site, but also by forming disulfide bridges between cysteines located in the vicinity of the nucleotide-binding sites. Comparing the effect of disulfiram on ATP hydrolysis in Pgp, MRP1, and the multidrug resistance protein 4 (MRP4 or ABCC4) has allowed us to elucidate these chemical interactions. Disulfiram thus has unique molecular interactions with both Pgp and MRP1 and plausibly with other proteins that mediate MDR or resistance to nucleoside analogs. Disulfiram is a drug with a long record of moderate side effects that has the potential of circumventing MDR in human cancers, which is associated with extremely high mortality (Gottesman et al., 2002).

Materials and Methods

Chemicals. Bodipy-verapamil and bodipy-FL-prazosin were purchased from Molecular Probes (Eugene, OR). Cyclosporin A was purchased from Calbiochem (San Diego, CA). [^{125}I]Iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]Azidopine (60 Ci/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [α-^{32}P]8-AzidoATP (15–20 Ci/mmol) and 8-azidoATP was purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). Pgp-specific monoclonal antibody C219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA), the polyclonal anti-MRP4 antibody was from Kamiya Biomedical (Thousand Oaks, CA), and the MRPr1 antibody was from Chemicon International (Temecula, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. The recombinant pcDNA3.1/MRP1-H_{10} plasmid containing the open reading frame of *MRP*1 was used to transfect HEK 293 cells in culture. Transfections were carried out using the LipofectAMINE 2000 reagent kit (Life Sciences, Gaithersburg, MD), according to the manufacturer's instructions. The stable transfectants were selected in geneticin (G418; 800 μg/ml) (Mediatech, Herndon, VA), and the selected clones were cultured in etoposide (5 μM). The cells were grown in the presence of G418 without the selection agent (etoposide) for 21 days before flow cytometry.

Preparation of Crude Membranes from High Five Insect Cells Infected with Recombinant Baculovirus Carrying the Human MDR1 or MRP4 cDNA. High Five insect cells (Invitrogen, Carlsbad, CA) were infected with the recombinant baculovirus carrying the human *MDR*1 cDNA with a six-histidine tag at the C-terminal end [BV-MDR1 (H6)] as described previously (Ramachandra et al., 1998) or with the human *MRP*4 cDNA (the plasmid pVL1393-MRP4 was provided by Dr. Gary Kruh, Fox Chase Cancer Center, Philadelphia, PA) (Lee et al., 2000). Crude membranes were prepared as described previously (Ramachandra et al., 1998). Intramolecular disulfide bonds between an active-site thiol and the thiol of another cysteine are the chemical basis of the inactivation by disulfiram (Shen et al., 2000). DTT (a component of the buffer in which the crude membrane preparations are stored) would reduce these disulfide bonds; thus, the membrane preparations were diluted 5-fold in DTT-free buffer and washed by centrifugation at 300,000*g* at 4°C for 10 min by using S120-AT2 rotor in an RC-M120EX microultracentrifuge (Sorvall, Newton, CT), and the pellet was resuspended in DTT-free buffer before conducting all experiments.

Preparation of Crude Membranes from HEK 293 Cells Stably Transfected with pcDNA3.1/MRP1-H_{10}. Crude membranes were prepared as described previously (Hrycyna et al., 1998), and stored at −70°C in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM sucrose, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1% aprotinin.

Fluorescent Drug-Accumulation Assay by Flow Cytometry. Fluorescent drug accumulation assay was performed in intact NIH-3T3 cells or in NIH-3T3 cells transfected with the human *MDR*1 gene (Cardarelli et al., 1995). Control or Pgp-expressing cells (250,000 per assay) were suspended in Iscove's modified Dulbecco's medium containing 5% fetal bovine serum. Cells were untreated or were exposed to the modulators at concentrations indicated in the figure legends for 5 min at 37°C. Bodipy-prazosin was then added to the samples at a final concentration of 0.5 μM and incubated at 37°C for an additional 40 min in the dark. All assays were carried out in a reaction mixture of 4.5 ml. At the end of the 40-min incubation, the cells were centrifuged for 10 min at 500*g* and transferred to ice. The cells were resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and analyzed on a FACSort flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

ATPase Assay. The Vi-sensitive ATPase activity of Pgp, MRP1, and MRP4 was measured by the inorganic phosphate (P_i) assay as described previously (Ramachandra et al., 1998; Sauna and Ambudkar, 2001). An important aim of this study is to distinguish between the effects of disulfiram on the ATP site and the drug-substrate site. To do this, we studied the effects of disulfiram when the ATP site was accessible or inaccessible to disulfiram. Figure 2 details the experimental strategy we devised. In principle, the ATP sites were protected by incubating the crude membranes in the presence of 10 mM ATP and 20 mM $MgCl_2$ at 4°C for 5 min before the addition of disulfiram. At 4°C, the ATP can bind to the ATP sites of the transport protein, but there is undetectable ATP hydrolysis [(the energetics of this reaction are detailed by Sauna et al. (2001a)]. ATP hydrolysis can then be initiated by transferring the samples quickly to 37°C.

Photoaffinity-Labeling of Pgp with IAAP. The crude membranes (10–50 μg protein) with the ATP site either free or protected (see above) were incubated with the drug or modulator for 3 min at room temperature in 50 mM Tris-HCl, pH 7.5, and IAAP (unless otherwise stated, 3–6 nM) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp assembly (PGC Scientifics, Gaithersburg, MD) fitted with

two black light (self-filtering) UV-A long-wave F15T8BLB tubes (365 nm) for 10 min at room temperature (21–23°C). After SDS-PAGE on an 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at −70°C for 12 to 24 h. The radioactivity incorporated into the Pgp band was quantified using the STORM 860 PhosphorImager system and the software ImageQuant (Amersham Biosciences Inc., Piscataway, NJ).

Photoaffinity-Labeling of Pgp with [^{3}H]Azidopine. The crude membranes were washed by centrifugation to remove DTT. Approximately 150 μg of the crude membranes were treated with increasing concentrations of disulfiram for 10 min at 37°C. The samples were brought to room-temperature and were treated with 0.5 μM [^{3}H]azidopine for 5 min and then photo–cross-linked over cold water at 365 nm for 10 min, and 5× SDS sample buffer was added. After electrophoresis, the gel was incubated with Fluoro-Hance (Research Products International Corporation, Mt. Prospect, IL) for 30 to 45 min and dried under vacuum, and the dried gel was exposed to an X-ray film for 4 to 6 days at −70°C.

Binding of [α-^{32}P]8-AzidoATP to Pgp. Crude membranes (1 mg/ml) were incubated in the ATPase assay buffer containing 10 μM [α-^{32}P]8-azidoATP (containing 10 μCi/nmol) in the dark at 4°C for 5 min in the presence or absence of indicated concentrations of disulfiram. The samples were then illuminated with a UV lamp assembly (365 nm) as described above for 10 min on ice (4°C). Ice-cold ATP (12.5 mM) was added to displace excess noncovalently bound [α-^{32}P]8-azidoATP. After SDS-PAGE on an 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film at −70°C for 12 to 24 h. The radioactivity incorporated into the protein (Pgp, MRP1, or MRP4) band was quantified as described above.

Induction of Disulfide Cross-Links by Cu^{2+} Oxidation and by Disulfiram. Crude membranes prepared from either High Five insect cells overexpressing Pgp or HEK 293 cells stably expressing MRP1 were washed by centrifugation and resuspended in DTT-free buffer. Membranes (100 μg protein) were incubated with 100 μM disulfiram or 3 mM $CuSO_4$ and 9 mM 1,10-phenanthroline for 10 min at 37°C (Urbatsch et al., 2001). The reaction was stopped by the addition of 40 mM EDTA. Each sample was divided into two aliquots: one was treated with SDS-PAGE sample buffer, and the other was treated with SDS-PAGE sample buffer in which β-mercaptoethanol was omitted. The Pgp, MRP1, or MRP4 proteins were identified on a Western blot using the monoclonal antibodies C219 (Kartner et al., 1985), MRPr-1 (Flens et al., 1994), or the polyclonal anti-MRP4 antibody (Schuetz et al., 1999), respectively.

Results

Disulfiram Reverses the Efflux of Drugs in NIH-3T3 Cells Expressing Pgp. The previous report on the use of disulfiram to circumvent drug resistance in cells expressing the *MDR*1 gene (Loo and Clarke, 2000a) argues that the mechanism(s) of action are the inhibition of Pgp activity via cysteine modification and/or by blocking the maturation of Pgp. The study explored the latter possibility in considerable detail and presented evidence that disulfiram does indeed impede the maturation of Pgp. The authors also demonstrated that disulfiram inhibits substrate-stimulated ATP hydrolysis by Pgp. Even if disulfiram does interact directly with Pgp, the phenomena can only be exploited clinically to circumvent Pgp-mediated MDR if it were demonstrated that disulfiram reverses drug efflux in intact cells. We have investigated the effect of disulfiram on the efflux of bodipy-prazosin and calcein-AM from Pgp-overexpressing cells. The NIH-MDR-G185 cells expressing the *MDR*1 gene show significantly reduced accumulation of bodipy-prazosin caused by efflux of the drug by Pgp. This can be reversed by the addition of cyclosporin A (a reversal agent) to obtain a level of bodipy-prazosin or calcein comparable with that in control cells. Disulfiram shows a reversal of bodipy-prazosin efflux comparable with that of cyclosporin A (Fig. 1A). Disulfiram, similarly, reverses the efflux of calcein-AM by Pgp (Fig. 1B). Control NIH-3T3 cells, however, show accumulation of both bodipy-prazosin and calcein, and this accumulation is not affected by cyclosporin A or disulfiram (data not shown). Moreover, consistent with previous work (Mechetner et al., 1997), Pgp shows a significant increase in the reactivity of the conformationally sensitive Pgp-specific antibody UIC2 in the presence of cyclosporin A (Fig. 1C). There is, moreover, a comparable increase in the UIC2 reactivity in the presence of disulfiram (Fig. 1C), suggesting that disulfiram effects a conformational change similar to that of other substrates such as cyclosporin A or vinblastine. Taken together, these results demonstrate that disulfiram is a modulator of transport by Pgp and that it directly interacts with the transporter.

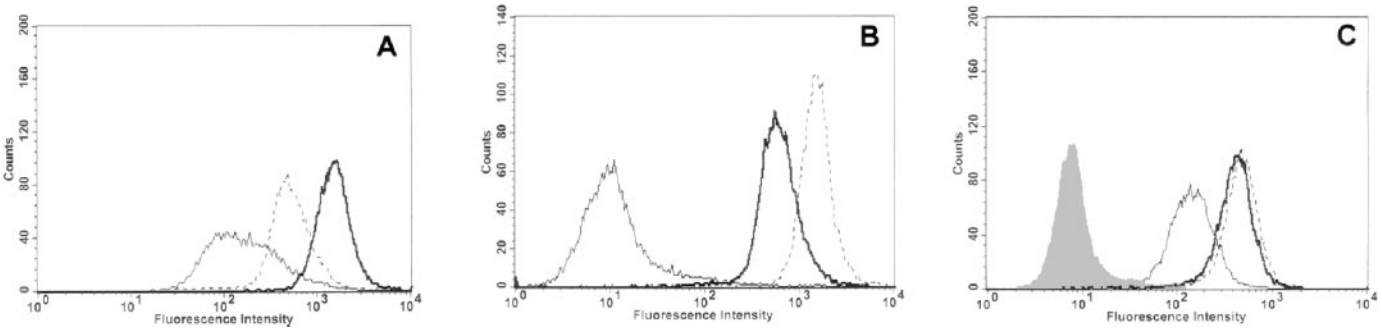

Fig. 1. Effect of disulfiram on transport of drug substrates by NIH-3T3 cells expressing the *MDR*1 gene and on the accessibility of the epitope for the human Pgp specific antibody UIC2. The effect of disulfiram was monitored in human *MDR*1-expressing NIH-3T3 cells by using Pgp substrates bodipy-FL-prazosin (A) or calcein-AM (B) in transport assays and by monitoring the increase in the binding of the Pgp-specific, conformationally sensitive monoclonal antibody UIC2 (C). The transport assays were performed using 0.5 μM bodipy-FL-prazosin or 0.25 μM calcein-AM and analyzed by flow cytometry as described under *Materials and Methods*. The UIC2 reactivity shift assay (Mechetner et al., 1997) was performed by pretreating the cells with 20 μM disulfiram or 5 μM cyclosporin A for 5 min at 37°C before incubation with UIC2 (1 μg/100,000 cells) for 30 min at 37°C in a reaction volume of 400 μl. The cell suspension was diluted to 5 ml with Iscove's modified Dulbecco's medium containing 5% fetal bovine serum and centrifuged at 500*g* for 5 min, and the pellet was resuspended in 400 μl of medium with fluorescein isothiocyanate-labeled anti-mouse IgG2a (1 μg/500,000 cells) and incubated for 30 min at 37°C in the dark. Cells were washed, resuspended in 400 μl of ice-cold phosphate-buffered saline and analyzed by flow cytometry. The histograms show untreated cells (thin line) and those treated with 5 μM cyclosporin A (dashed line) or 20 μM disulfiram (heavy line). The gray-filled histogram in C represents the signal from cells treated with IgG2a isotype control antibody instead of UIC2. These figures are representative of three independent experiments.

The extent of disulfiram-mediated reversal of drug efflux is comparable with that effected by cyclosporin A, and the level of accumulation of bodipy-prazosin is comparable with that observed in control NIH-3T3 cells. However, this observation is qualitative. We therefore monitored the reversal of bodipy-prazosin efflux in the NIH-MDR-G185 cells in the presence of increasing concentrations (1–100 μM) of disulfiram and used the median fluorescence at each concentration of disulfiram to generate a dose-response curve (data not shown) to determine that disulfiram has an EC_{50} of 17.8 $\pm$ 2.1 μM (n = 3). An understanding of the mechanism by which disulfiram effects the reversal of Pgp-mediated drug efflux would be useful in the clinical development of disulfiram.

Disulfiram Inhibits the Basal ATP Hydrolysis by Pgp, but if the ATP Sites Are Protected, It Stimulates Hydrolysis. The transport of drug substrates by Pgp is coupled to ATP hydrolysis, and there is evidence of drug-stimulated ATPase activity in Pgp from diverse systems (Sauna et al., 2001b). This has led to the use of the stimulation of ATP hydrolysis by Pgp as a surrogate assay to determine drug-Pgp interactions. Disulfiram has the potential to interact with Pgp in two different ways. From the fact that disulfiram inhibits the ATPase activity of wild-type Pgp but stimulates that of Cys-less mutant Pgp (in which all of the seven cysteines have been replaced with alanine) (Loo and Clarke, 2000a), it has been suggested that disulfiram modifies the critical cysteines in the Walker A domain of the ATP sites of Pgp (C431 and C1074). The data presented in Fig. 1 demonstrate that disulfiram reverses drug efflux by cells that overexpress Pgp. Thus, it is plausible that disulfiram competes for the drug-binding site(s). The experimental strategy depicted in Fig. 2 would allow one to ascertain whether disulfiram effects Pgp function by competing for the substrate binding sites, by modifying the ATP sites, or both. If the ATP sites were protected by adding excess ATP before the addition of disulfiram, the drug would be able to interact only with the drug-binding site(s). If, on the other hand, Pgp is allowed to interact with disulfiram before adding ATP, the disulfiram would covalently modify the ATP sites, and it will also interact with the substrate binding sites. Because this is an irreversible reaction, the ATP sites would be permanently disabled. The results of this experiment are shown in Fig. 3A. When the ATP sites were accessible, the disulfiram showed inhibition of the ATP hydrolysis by Pgp, possibly via the covalent modification of the cysteines. However, when the ATP sites were protected, disulfiram stimulated the ATP hydrolysis activity of Pgp. Thus, it would seem that disulfiram might be able to interact with both the drug binding and ATP sites of Pgp. For this assertion to be valid, it is important to demonstrate that the modification of cysteines in Pgp other than in the ATP site does not stimulate the ATP hydrolysis by Pgp. To do this, we compared the effect of disulfiram with a sulfhydryl-specific modifier, NEM, that is not a substrate of Pgp (al-Shawi et al., 1994; Urbatsch et al.,

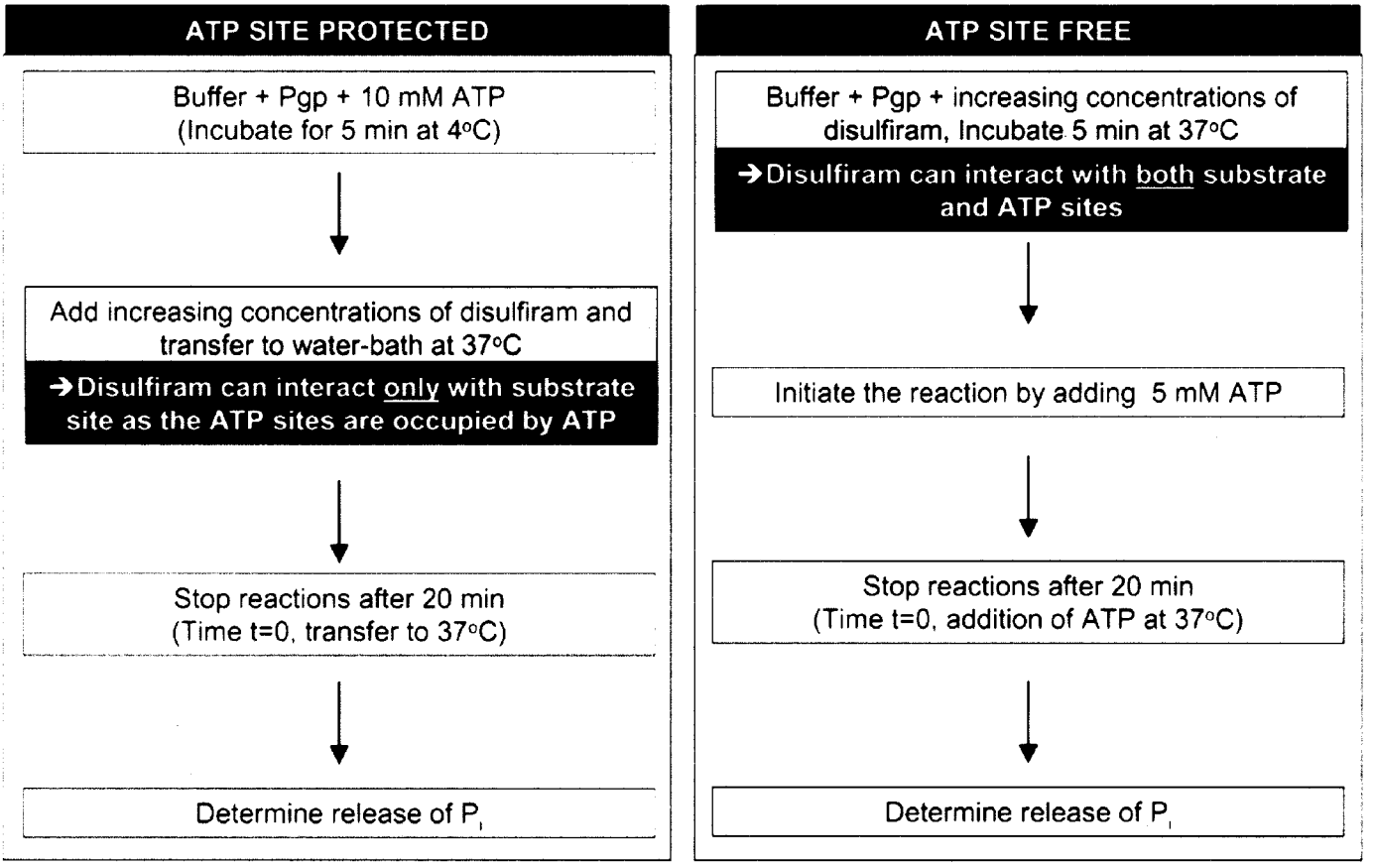

Fig. 2. Schematic describing an experimental strategy to distinguish the interaction of disulfiram with the drug-substrate and ATP sites of P-glycoprotein. Left, an experimental strategy to protect the ATP sites by allowing ATP under saturating conditions to bind at 4°C before the addition of disulfiram. As ATP occupies the ATP sites they are inaccessible to disulfiram. Right, if disulfiram is allowed to interact with Pgp before the addition of ATP, both substrate and ATP sites are accessible to disulfiram.

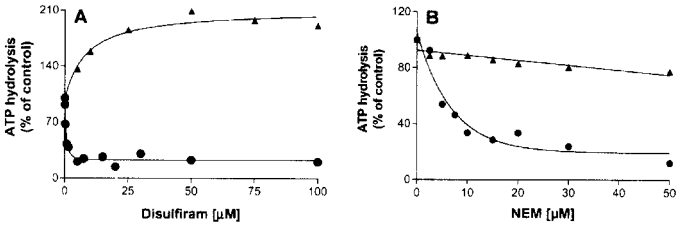

Fig. 3. Effect of disulfiram and *N*-ethylmaleimide on the ATP hydrolysis by Pgp. The Vi-sensitive ATPase activity of Pgp was determined by using the P_i release assay as described under *Materials and Methods* in the presence of increasing concentrations of disulfiram (A) or NEM (B). The assays were performed under conditions that either protected the ATP sites (▲) or allowed them to be accessible (●) to disulfiram or NEM (see Fig. 2 for description of the experimental conditions). The values were normalized such that ATP hydrolysis in the absence of disulfiram or NEM was equal to 100%. The data presented here are typical of four independent experiments. The IC_{50} and K_m (mean ± S.D.) values for the inhibition and stimulation of ATP hydrolysis by disulfiram were 1.2 ± 0.06 μM (n = 4) and 8.34 ± 1.6 μM (n = 4), respectively.

1994). We show in Fig. 3B that with the ATP sites unprotected, NEM inhibits the ATP hydrolysis by Pgp, and its effect is comparable with that of disulfiram. On the other hand, when the ATP sites were protected, NEM had no effect on the ATP hydrolysis by Pgp.

Disulfiram Inhibits the Binding of IAAP and Azidopine to Pgp. The experiment depicted above suggests that disulfiram may interact directly with the substrate binding site(s) of Pgp. Using substrate analogs that photoaffinity-label Pgp (Sauna et al., 2001b) offers the most direct experimental strategy to address the issue of whether disulfiram interacts with the substrate binding site. The experiments presented in Fig. 1A use bodipy-prazosin as a fluorescent analog of the Pgp substrate prazosin. To be consistent, we used the ^{125}I-iodoarylazido derivative of prazosin, IAAP, for the photoaffinity studies. Previous studies have shown that IAAP specifically binds to Pgp with a $K_d \sim 400$ nM and that this binding competes with that of other substrates of Pgp (Sauna and Ambudkar, 2000). Moreover, IAAP is itself a transport substrate of Pgp in intact cells (Maki et al., 2003). We demonstrate (Fig. 4) that disulfiram inhibits IAAP binding to Pgp in a concentration-dependent manner, regardless of whether the ATP sites are free or protected (IC_{50} = 3.5 ± 0.43 and 6.3 ± 0.47 μM, respectively). A Michaelis-Menten model fits these data, suggesting that disulfiram may interact directly with the substrate binding site of Pgp. It is noteworthy that NEM has no effect on IAAP binding, although its effect on ATP hydrolysis by Pgp (with ATP sites

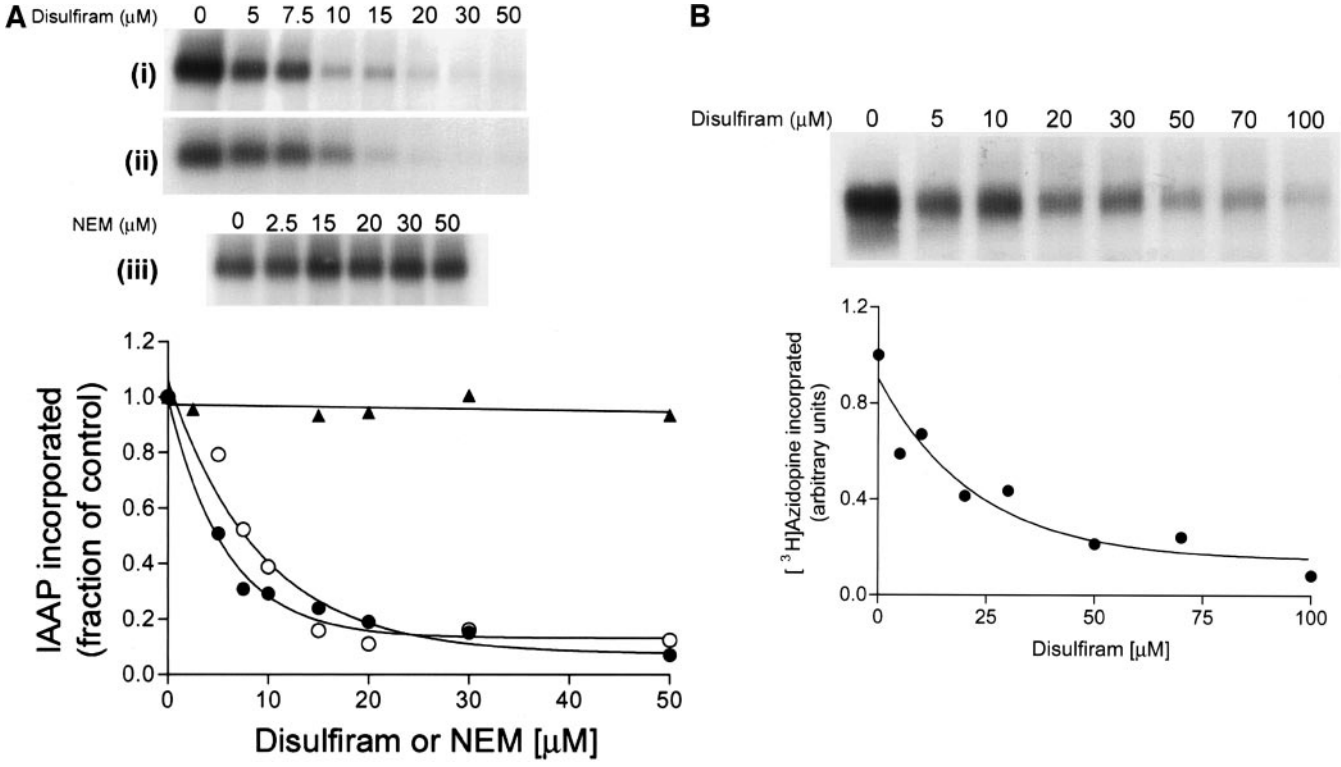

Fig. 4. Effect of disulfiram and NEM on the binding of photoaffinity analogs of Pgp drug-substrates. A, the binding of IAAP (5–10 nM) to Pgp was monitored as described under *Materials and Methods* to determine the effect of 1) increasing concentrations of disulfiram with the ATP sites protected (○); 2) increasing concentrations of disulfiram with the ATP sites free (●); and 3) increasing concentrations of NEM with the ATP sites free (▲). See Fig. 2 for a description of experimental conditions. After SDS-PAGE on a 8% Tris-glycine gel at constant voltage, gels were dried, and the radioactivity incorporated into the Pgp band was quantified using the STORM 860 PhosphorImager system and the software ImageQuant. The lines represent the best fit for the data by nonlinear least-squares regression analysis using Prism software (GraphPad Software Inc., San Diego, CA). The curves represent the data on the autoradiogram and are typical of three independent experiments. The IC_{50} values (mean ± S.D.) for inhibition of IAAP binding by disulfiram were 3.5 + 0.43 μM (n = 3) when the ATP sites were free and 6.3 + 0.47 μM (n = 3) when the ATP sites were protected. B, the binding of [^{3}H]azidopine (0.5 μM) to Pgp was monitored after treatment with increasing concentrations of disulfiram as described under *Materials and Methods*. After SDS-PAGE, the gel was treated with Fluoro-Hance (Research Products International Corporation), dried, and then exposed to an X-ray film, and the concentrations of disulfiram are shown on the autoradiogram. The radioactivity incorporated into the Pgp bands was quantified by densitometry, and the data were plotted using the curve-fitting Prism software. The IC_{50} (mean ± S.D.) for disulfiram-mediated inhibition of [^{3}H]azidopine binding to Pgp was 15.5 ± 4.3 μM (n = 3).

free) is comparable with that of disulfiram (compare Figs. 4A and 3B).

To demonstrate that the inhibition of drug-substrate binding by disulfiram is not specific to IAAP, we performed the same experiment with another photoaffinity analog, the dihydropyridine [^{3}H]azidopine. The results depicted in Fig. 4B demonstrate that disulfiram inhibits the binding of [^{3}H]azidopine to Pgp in a concentration-dependent manner with an IC_{50} (15.5 ± 4.3 μM) comparable with that of IAAP. Thus, it seems that disulfiram interacts directly with the drug-substrate binding site of Pgp.

Disulfiram Inhibits the Binding of [α-^{32}P]8-AzidoATP to Pgp. Effects on substrate-stimulated ATP hydrolysis can be complex and could occur at different steps in the catalytic cycle. To directly assess whether disulfiram effects the binding of nucleotide to Pgp, we monitored the binding of [α-^{32}P]8-azidoATP to Pgp in the presence of increasing concentrations of disulfiram (Fig. 5A). The IC_{50} (disulfiram) for inhibition of [α-^{32}P]8-azidoATP binding (8.1 ± 1.03 μM) is in the same range as the IC_{50} for the inhibition of IAAP binding (3.5–6.3 μM). Moreover, whereas NEM has no effect on the binding of IAAP to Pgp (Fig. 4A), it inhibits [α-^{32}P]8-azidoATP binding with an IC_{50} of 7.3 ± 0.87 μM (Fig. 5B), which is comparable with the IC_{50} for disulfiram. Finally, we analyzed the effect of disulfiram on the substrate-stimulated ATP hydrolysis by Pgp (Table 1) using diverse Pgp substrates. We found that disulfiram inhibits ATP hydrolysis in the presence of the drugs valinomycin (10 μM), vinblastine (5 μM), reversin (0.1 μM), tertramethylrhodamine (5 μM), and bodipy-verapamil (5 μM). The IC_{50} (disulfiram) for inhibition of the substrate-stimulated ATP hydrolysis by different drugs is in the range of 25 to 55 μM. This is additional evidence that disulfiram acts as an inhibitor of the binding of substrates to Pgp.

Effect of Disulfiram on MRP1. The data presented above clearly show that disulfiram interacts with Pgp. An important question thus would be whether disulfiram interacts specifically with Pgp or also interacts with other ABC transport proteins implicated in MDR. Figure 6 demonstrates that in HEK 293 cells overexpressing the MRP1 gene, the efflux of both fluo 4-AM and calcein-AM is reversed by disulfiram to the same extent as that of MK571, an inhibitor specific to MRP1. Disulfiram reverses the efflux of calcein-AM with an EC_{50} = 26.8 ± 3.4 μM (n = 3), which is comparable with the EC_{50} (disulfiram) for Pgp-mediated efflux of bodipy-prazosin. Moreover, disulfiram also inhibits ATP hydrolysis by MRP1 in a dose-dependent manner. However, if the ATP sites of MRP1 are protected with excess ATP

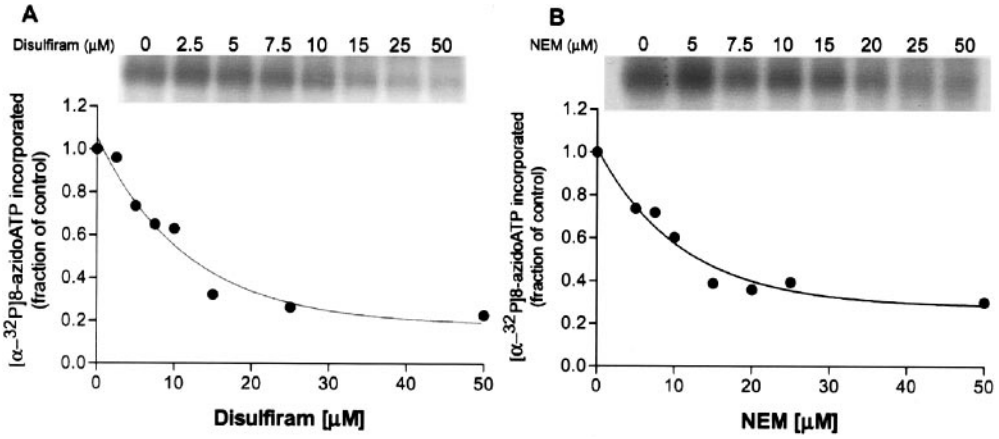

Fig. 5. The effect of disulfiram and NEM on the binding of [α-^{32}P]8-azidoATP to Pgp. The binding of [α-^{32}P]8-azidoATP (10 μM, 10 μCi/nmol) was determined as described under *Materials and Methods* at 4°C (under nonhydrolysis conditions) in the presence of increasing concentrations of disulfiram (A) or NEM (B). The [α-^{32}P]8-azidoATP incorporated into the Pgp band was quantified as described in the legend to Fig. 4A. The lines represent the best fit for the data by nonlinear least-squares regression analysis using Prism software. The concentrations of disulfiram or NEM are given on the autoradiograms in both A and B. The curves represent quantification of the autoradiogram that is depicted and are typical of three independent experiments. The IC_{50} values (mean ± S.D.) for inhibition of [α-^{32}P]8-azidoATP binding by disulfiram and NEM were 8.1 ± 1.03 and 7.3 ± 0.87 μM (n = 3), respectively.

TABLE 1

Kinetics of the inhibition by disulfiram of the drug-stimulated Vi-sensitive ATPase activity of Pgp

Values represent the mean (n = 3) ± S.D. The effect of disulfiram was monitored at a fixed concentration (given in parentheses) of a given agent as indicated. The Vi-sensitive ATPase activity or Pgp in the absence of substrate or modulator (Basal) or in the presence of fixed concentration of indicated drug in the absence of disulfiram (Stimulated) was determined as described under *Materials and Methods*. Stimulation of Vi-sensitive ATPase activity of Pgp by the drug in the absence of disulfiram is expressed as mean fold ± S.D. ATP hydrolysis was monitored in the presence of fixed concentration of the drug and increasing concentrations of disulfiram. IC_{50} values were obtained by using the curve-fitting software GraphPad.

Drug	ATPase Activity		Fold Stimulation	IC_{50}
	Basal	Stimulated	(ATPase activity)	(Disulfiram)
	nmol P_i/min/mg of protein			*μM*
Valinomycin (10 μM)	24.59 ± 1.27	125.78 ± 5.58	5.26 ± 1.11	25.38 ± 3.65
Vinblastine (5 μM)	23.49 ± 1.85	86.80 ± 1.35	3.71 ± 0.35	31.15 ± 2.19
Reversin (0.1 μM)	25.39 ± 0.16	101.47 ± 0.50	4.00 ± 0.05	25.10 ± 2.26
Tertramethylrhodamine (5 μM)	22.08 ± 0.96	110.18 ± 4.14	4.99 ± 0.03	53.85 ± 1.97
Bodipy verapamil (5 μM)	24.68 ± 2.22	171.11 ± 10.99	7.00 ± 1.03	41.33 ± 6.77

(see Fig. 2 for explanation of the experimental strategy), there is a small increase in the ATP hydrolysis and then inhibition at higher concentrations (Fig. 7A). This is at variance with the phenomenon observed in Pgp, in which there is no inhibition of ATP hydrolysis when the ATP sites are protected (Fig. 3). The chemical basis of inhibition by disulfiram could provide an explanation for this discrepancy (see below). Finally, consistent with these results, we demonstrate that disulfiram inhibits the binding of [α-^{32}P]8-azidoATP to MRP1 with a IC_{50} of 20 $\pm$ 1.6 μM (Fig. 7B).

The Chemical Basis of the Inhibitory Effects of Disulfiram on ATP Hydrolysis. The data presented above show that excess ATP protects Pgp completely from the inhibitory effects of disulfiram, but it protects MRP1 only partially. The Walker A domains of both ATP sites of Pgp contain cysteine residues, whereas only the N-ATP site of MRP1 has a cysteine. However, overall, MRP1 has 25 cysteines (Cole et al., 1992), whereas Pgp has only seven cysteine residues (Chen et al., 1986). Moreover, it has been established in aldehyde dehydrogenase that disulfiram first interacts with an active-site cysteine to form an unstable intermediate that can interact with the thiol groups of surrounding cysteines (Fig. 8). Consistent with this scheme, radiolabeled NEM migrates with the Pgp band because it is covalently linked to the protein (al-Shawi et al., 1994). However, disulfiram participates in generating an intramolecular disulfide bond but is not itself part of the final product; as such, it cannot be used to cross-link or label transport proteins. We exploited the fact that human MRP4 (ABCC4) has no cysteines in the Walker A and B domains of either the N- or C-ATP sites, although there are a total of 15 cysteine residues (Lee et al., 1998), in this transport protein to experimentally verify the chemical interaction between disulfiram and cysteines in a protein. In such a protein, NEM, which modifies only a single cysteine, would not inhibit ATP binding because there are no cysteines in the active site. Figure 9A shows that NEM, at concentrations as high as 100 μM, does not inhibit the binding of [α-^{32}P]8-azidoATP to MRP4. There is, however, a significant inhibition by disulfiram that can potentially inhibit nucleotide binding by forming disulfide linkages across neighboring cysteines. Thus, disulfiram-mediated inhibition of ATP hydrolysis can occur even if the protein lacks a cysteine in the active site. Consistent with this postulate, we find that disulfiram inhibits ATP hydrolysis by MRP4 and that excess ATP cannot completely protect from this inhibition (Fig. 9B).

The chemical basis of modification by disulfiram in this mechanism suggests an irreversible intramolecular disulfide bond. It has been demonstrated previously that intramolecular disulfide cross-links can be induced in Pgp by Cu^{2+} oxidation (Loo and Clarke, 2000b; Urbatsch et al., 2001). The cross-linked species of Pgp runs as a higher molecular weight species when reducing agents such as β-mercaptoethanol and DTT are omitted from the SDS-PAGE sample buffer. Figure

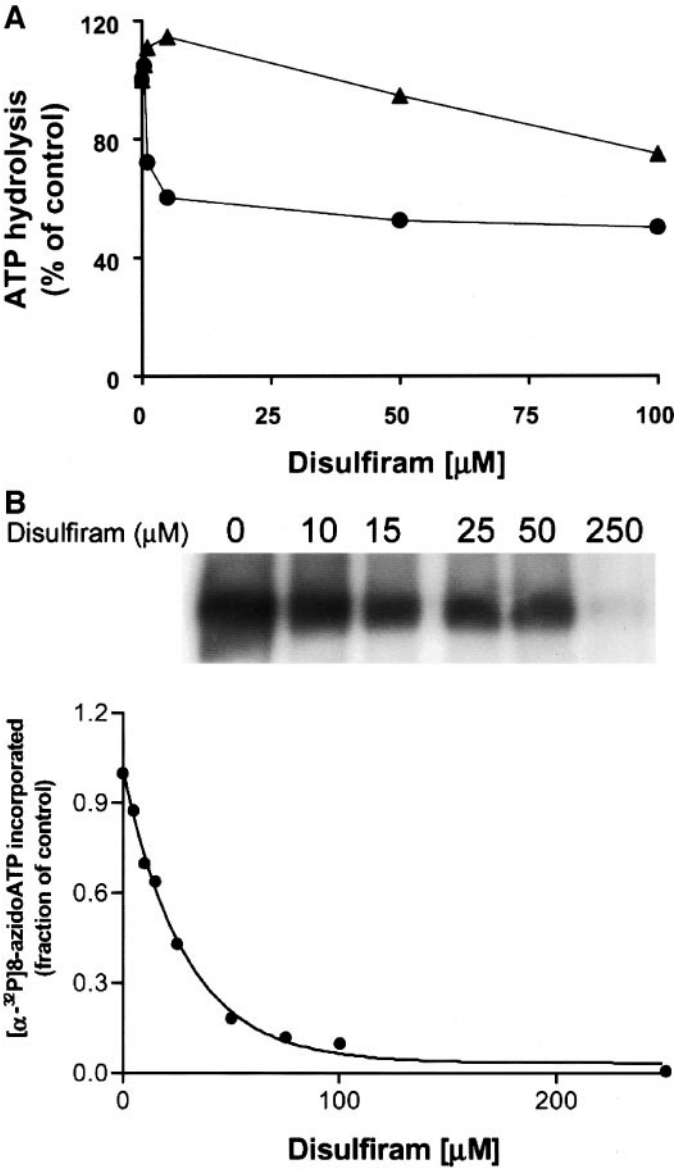

Fig. 7. Effect of disulfiram on ATP hydrolysis by and [α-^{32}P]8-azidoATP-binding to MRP1. A, the Vi-sensitive ATPase activity of MRP1 was determined by using the P_i release assay as described under *Materials and Methods* in the presence of increasing concentrations of disulfiram. The assays were performed under conditions that either protected the ATP sites (▲) or allowed them to be accessible (●) to disulfiram (see Fig. 2 for a description of the experimental conditions). Data were normalized such that ATP hydrolysis in the absence of disulfiram was equal to 100%. B, the binding of [α-^{32}P]8-azidoATP (10 μM, 10 μCi/nmol) to MRP1 was determined as described under *Materials and Methods* at 4°C (under nonhydrolysis conditions) in the presence of increasing concentrations of disulfiram. The [α-^{32}P]8-azidoATP incorporated into the MRP1 band was quantified as described in the legend to Fig. 4 A. The lines represent the best fit for the data by nonlinear least-squares regression. Values shown in the graph are derived from the autoradiogram (top). IC_{50} for disulfiram-mediated inhibition of binding of [α-^{32}P]8-azidoATP to MRP1 is 20 $\pm$ 1.6 μM (n = 3).

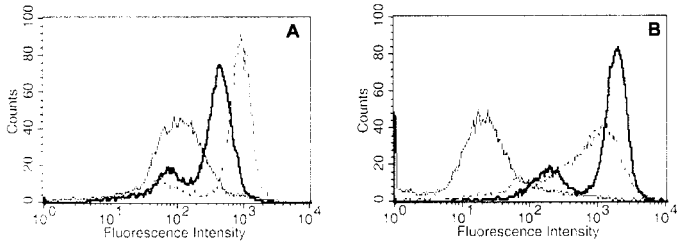

Fig. 6. Effect of disulfiram on transport of drug substrates by HEK 293 cells expressing the MRP1 gene. The transport assays were performed in HEK 293 cells expressing the MRP1 gene using 0.5 μM fluo 4-AM (A) or 0.25 μM calcein-AM (B) and analyzed by flow cytometry as described under *Materials and Methods*. The data presented as histograms show untreated cells (thin line) and those treated with 20 μM MK571 (dashed line) or 50 μM disulfiram (heavy line).

10A shows that treatment with disulfiram generated the higher molecular weight species similar to that generated by Cu^{2+} treatment. Moreover, a similar higher molecular weight species was observed when MRP1 was treated with disulfiram (Fig. 10B).

Discussion

Tumor cells that exhibit Pgp-mediated MDR pose a significant clinical problem (Gottesman et al., 2002). Most compounds that are used to reverse the activity of Pgp are competitive inhibitors (Tan et al., 2000). Recently, a novel approach to reverse Pgp-associated MDR was advocated using the drug disulfiram (Loo and Clarke, 2000a). The authors presented evidence that disulfiram prevents the maturation of Pgp and thus reduces Pgp-mediated MDR. They also showed preliminary evidence that disulfiram interacts directly with purified Pgp, inhibiting the substrate-stimulated ATPase activity of Pgp presumably via cysteine modification.

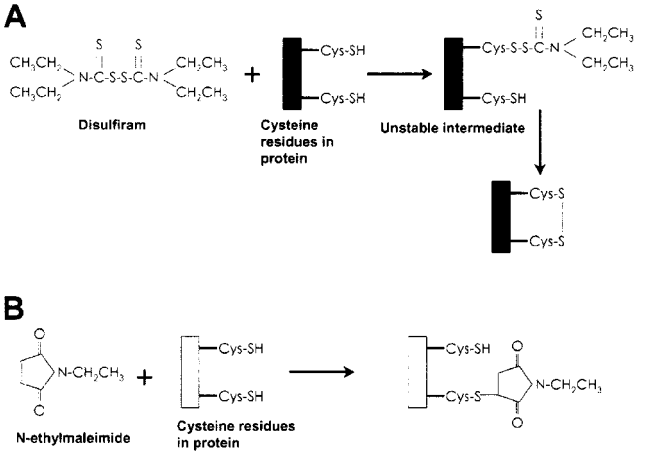

Fig. 8. Comparison of the chemical reaction of disulfiram and NEM with cysteine residues in proteins. Possible mechanisms for the interaction of cysteine residues in proteins are shown with disulfiram (A) and NEM (B). Disulfiram first interacts with a cysteine residue to form an unstable mixed disulfide. If there are additional cysteine moieties in the vicinity of the unstable intermediate, it reacts with the thiol group to form an intramolecular disulfide bond [Shen et al. (2000) gives a detailed mechanism]. There is no such intramolecular disulfide generated with NEM.

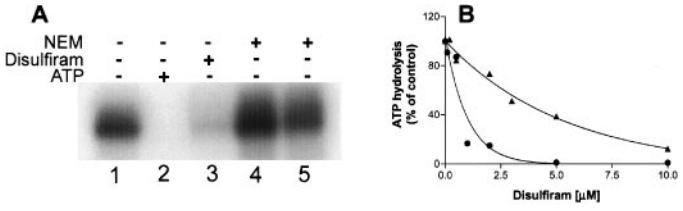

Fig. 9. Effect of disulfiram and NEM on the binding of [α-^{32}P]8-azidoATP to MRP4 and on MRP4-mediated ATP hydrolysis. A, the binding of [α-^{32}P]8-azidoATP (10 μM, 10 μCi/nmol) was determined as described under *Materials and Methods* at 4°C (under nonhydrolysis conditions) in the presence of dimethyl sulfoxide (control) (lane 1), 10 mM ATP (lane 2), 50 μM disulfiram (lane 3), 50 μM NEM (lane 4), or 100 μM NEM (lane 5). The treatments are described on the figure. B, the Vi-sensitive ATPase activity of MRP4 was determined in crude membranes of High Five insect cells (100 μg protein/ml) by using the P_i release assay as described under *Materials and Methods* in the presence of increasing concentrations of disulfiram. The assays were performed under conditions that either protected the ATP sites (▲) or allowed them to be accessible (●) to disulfiram (see Fig. 2 for a description of the experimental conditions). Data were normalized such that ATP hydrolysis in the absence of disulfiram was equal to 100%.

In this study we demonstrate the following: 1) disulfiram inhibits Pgp- and MRP1-mediated efflux of fluorescent drug-substrates from intact cells; 2) disulfiram is unique among MDR modulators in that it interacts with both the ATP and drug-substrate binding sites of Pgp and MRP1; 3) the ATP-site is incapacitated via the formation of disulfide bonds across neighboring cysteines; 4) disulfiram interacts directly at the drug-substrate site; and 5) disulfiram modulates MDR at concentrations that can be achieved in vivo and at levels far below the toxicity limits.

We used flow cytometry to evaluate whether disulfiram can inhibit the transport function of Pgp in intact cells. It was clear that in *MDR*1-expressing NIH-MDR-G185 cells, disulfiram reverses the efflux of fluorescent drugs bodipy-FL-prazosin or calcein-AM by Pgp (Fig. 1). Similarly, disulfiram was also found to block the efflux of fluo 4-AM and calcein-AM mediated by MRP1 in HEK 293 stable transfectants (Fig. 6). On evaluating the potency of disulfiram, we found that disulfiram has an EC_{50} value of 17.8 ± 2.1 μM (n = 3), which is achievable in a clinical setting. Similarly, the EC_{50} value for the inhibition of calcein-AM efflux mediated by MRP1 was 26.8 ± 3.4 μM (n = 3).

Disulfiram could modulate transport in intact cells via several mechanisms: 1) disulfiram could interact directly with the substrate binding site and arrest drug transport by acting as a competitive inhibitor; 2) it could modify the functionally important cysteines in the ATP sites, preventing ATP hydrolysis and limiting the energy source for drug transport; and 3) it is plausible that modification of cysteines effects conformational changes in the substrate binding site, preventing drug binding by an allosteric mechanism. We have experimentally evaluated each of these possibilities.

To understand what influence the modification of cysteines has on the ATP sites of Pgp and MRP1, we used NEM, which has been extensively used as a cysteine-modifying agent in protein chemistry and has been previously shown to inhibit ATP hydrolysis by Pgp (al-Shawi et al., 1994; Urbatsch et al., 1994). We show that when the ATP sites are accessible, the basal ATP hydrolysis of Pgp and MRP1 in the absence of any substrate is inhibited by both disulfiram and NEM by approximately 90% (Figs. 3 and 7). Interestingly, however, if the ATP sites of Pgp are protected, disulfiram stimulates ATP hydrolysis (Fig. 3A), whereas NEM has no effect (Fig. 3B). MRP1, however, shows only a small stimulation of ATP hydrolysis followed by inhibition by disulfiram when the ATP sites are protected. When the ATP sites are protected, agents that only modify the cysteines (such as NEM) would not be expected to exert any influence on the ATP sites. In addition, this control demonstrates that ATP adequately protects against cysteine modifications in the active site. Disulfiram stimulates ATP hydrolysis under these conditions, suggesting that disulfiram interacts at a substrate site of Pgp. Why then does MRP1 show inhibition of ATP hydrolysis at higher concentrations of disulfiram even when the ATP sites are protected? The chemistry of the interaction of disulfiram with cysteines and the distribution of the cysteines in MRP1 may provide an explanation. As illustrated in Fig. 8, disulfiram can form disulfide bridges across two cysteines that lie within a distance of approximately 8 Å. Overall, Pgp has far fewer cysteines than MRP1 (7 versus 25). However, Pgp has a cysteine in the Walker A domains of both the N- and C-terminal ATP sites, whereas MRP1 has a cysteine only in

the Walker A domain of the N-terminal ATP site. Thus, ATP can fully protect the cysteines in both ATP sites of Pgp. However, because of the large number of cysteines in MRP1, it is plausible that disulfiram could inhibit ATP hydrolysis by forming disulfide bridges across cysteines in the vicinity of, although not actually at, the ATP site. The cysteines in the intracellular cytoplasmic region would be candidates for such disulfide bridges. MRP1 has 10 cysteines located in the cytoplasmic region, whereas Pgp has only four. The modification of such cysteines would not be fully protected by ATP. To determine whether it is possible to inhibit ATP hydrolysis by such a mechanism, we used MRP4 (ABCC4), which has no cysteines in the conserved Walker A or B or the signature region of either the N- or C-ATP site but contains a total of 15 cysteines, of which nine are in the intracellular cytoplasmic region (Lee et al., 1998). We demonstrate that disulfiram can indeed inhibit binding of [α-^{32}P]8-azidoATP to MRP4, whereas NEM cannot (Fig. 9A). Additionally, the inhibition of ATP hydrolysis in MRP4 by disulfiram cannot be fully protected by ATP (Fig. 9B). Finally, we directly show evidence of intra- and intermolecular cross-linking by disulfiram in Pgp and MRP1 using a gel-mobility assay (Fig. 10).

When the ATP sites are protected, disulfiram stimulates ATP hydrolysis in Pgp (and to a limited extent in MRP1), suggesting that besides the modification of cysteines, disulfiram may interact with the drug-substrate site(s). Direct evidence that disulfiram competes for the substrate binding site of Pgp is presented in Fig. 4. The treatment of Pgp with NEM has no effect on IAAP binding, which is consistent with a previous report demonstrating that NEM could not displace the binding of [^{3}H]vinblastine to Pgp (Martin et al., 2000). On the other hand, an equivalent treatment with disulfiram reduces IAAP binding by more than 90% (Fig. 4A). Furthermore, disulfiram shows a similar inhibition of binding of another photoaffinity analog, [^{3}H]azidopine. NEM does not inhibit the binding of IAAP to disulfiram (Fig. 4A). On the other hand, both disulfiram and NEM inhibit the binding of [α-^{32}P]8-azidoATP to Pgp with IC_{50} values of 8.1 $\pm$ 1.03 and 7.3 $\pm$ 0.87 μM, respectively. These results suggest that the inhibition of IAAP binding occurs by a mechanism distinct from the effects of cysteine modification by disulfiram. We suggest that disulfiram blocks drug transport by Pgp in a two-pronged manner: as an inhibitor that competes for the drug-binding site and as a cysteine-modifying reagent that inactivates essential cysteines in the nucleotide-binding pocket to arrest ATP hydrolysis. In intact cells, the intracellular concentrations of ATP are in the range of 3 to 5 mM, and it could be argued that the ATP sites would be protected and not be modified by disulfiram. However, as illustrated in Fig. 8, disulfiram is an atypical cysteine-modifying agent and forms disulfide bridges between cysteines that lie within ~8 Å of each other. Thus, active sites that do not possess cysteines but are surrounded by two or more cysteines would be particularly vulnerable even in the presence of high intracellular concentrations of ATP. We illustrate this experimentally in Figs. 7A and 9B. The ATP hydrolysis by ABC proteins that possess large numbers of cysteines around but not in the active site (see above) is sensitive to disulfiram even in the presence of excess ATP. Another very powerful technique that has been developed in recent years is the ex vivo expansion of antigen-specific cells to induce antitumor immune responses (Dudley and Rosenberg, 2003). These ex vivo techniques have considerable potential in the treatment of hematological malignancies. MDR is a significant clinical problem in the treatment of several hematological malignancies such as acute myeloid and lymphoblastic leukemias and multiple myelomas (Sonneveld, 2000). Thus, the selective ATP depletion of such cells making them more susceptible to disulfiram

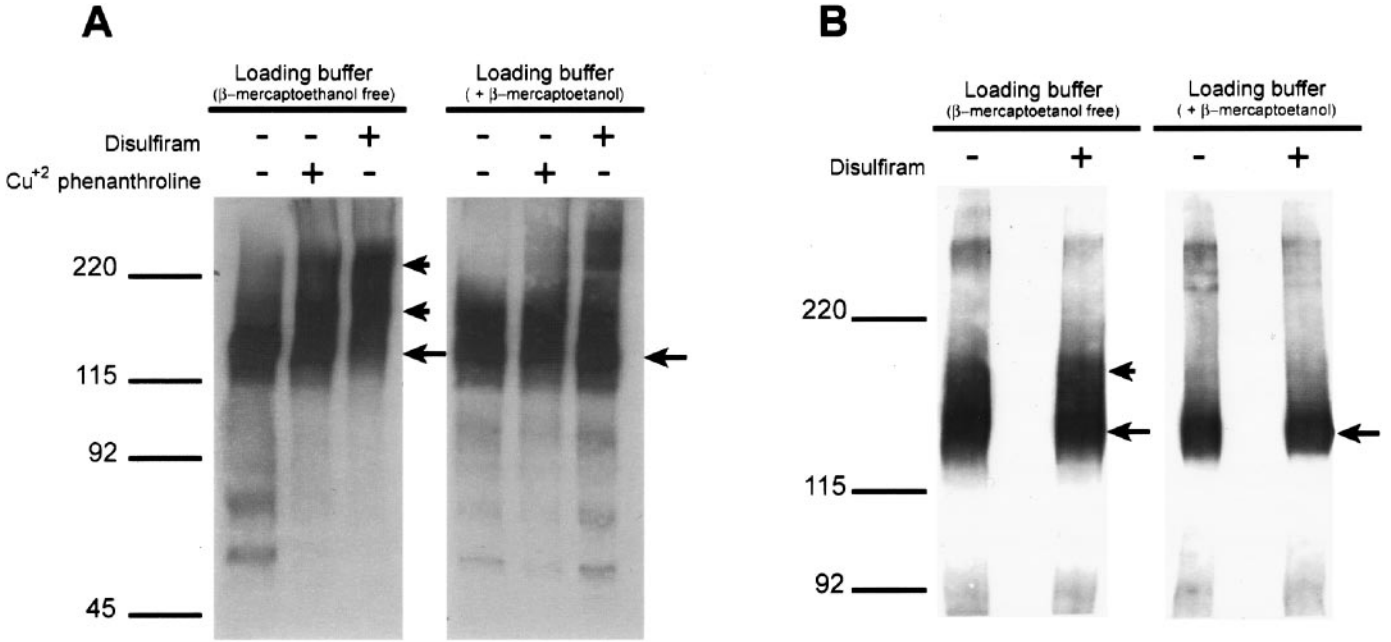

Fig. 10. Disulfiram induces disulfide cross-links in both Pgp and MRP1. Crude membranes prepared from either High Five insect cells overexpressing Pgp (A) or HEK 293 cells stably expressing MRP1 (B) were washed by centrifugation and resuspended in DTT free buffer and treated with 100 μM disulfiram or 3 mM $CuSO_4$ and 9 mM 1–10-*ortho*-phenanthroline (Urbatsch et al., 2001). The membranes were then processed as described under *Materials and Methods*. The experimental conditions are described on the figure. Arrows, Pgp or MRP1 band; arrowheads, the cross-linked species. The Western blot with C219 in A and MRPr-1 in B is representative of three independent experiments.

is also a possibility that may be technologically viable. Our results therefore suggest that for the reversal of Pgp- and MRP1-mediated MDR in a clinical setting, the interactions of disulfiram with both the drug-substrate and ATP binding site can potentially be exploited.

The EC_{50} value for disulfiram is at least an order of magnitude higher than that for cyclosporin A and several other new compounds currently in development (Tan et al., 2000). Thus, it may be argued that disulfiram is akin to the failed first-generation modulators of Pgp, which were originally developed for other disease conditions and for reversing MDR only at relatively high concentrations. However, the extremely low toxicity of disulfiram (Chick, 1999) coupled with the fact that sulfhydryl-reacting agents, despite their apparently unspecific mode of action, provide useful drugs against human diseases (Yakisich et al., 2001; Scozzafava et al., 2002) distinguishes it from other first-generation MDR modulators. Also, disulfiram has been in clinical use for several decades and has a well-documented pharmacology. Also, it must be borne in mind that disulfiram is unique in that as a single chemical moiety it neutralizes the effect of both Pgp and MRP1 by three independent mechanisms at two different levels, as discussed above. In addition, because Pgp and MRP1 are strongly implicated in MDR and the associations between MRP4 and resistance to antiviral nucleotide analogs is characterized (Lee et al., 2000), the pharmacologic effects of disulfiram are interesting. In this study, MRP4 was primarily used to understand the chemical basis of how disulfiram disables the ATP sites of ABC transporters. However, MRP4 is a clinically important molecular pump in its own right, which has been shown to transport cyclic nucleoside monophosphates and nucleoside analog drugs used in both anticancer and antiviral therapy as well as anticancer agents such as methotrexate, cladribine, and gemcitabine (Reid et al., 2003). Studies to characterize the interactions of disulfiram with MRP4 and other members of the MRP family (ABC C subgroup) are thus under way.

Acknowledgments

We thank Drs. Michael Gottesman, Tito Fojo, and Susan Bates for helpful discussions and Dr. Gary Kruh (Fox Chase Cancer Center) for providing MRP4 plasmid (pVL1393-MRP4). We also thank the National Cancer Institute Fellows Editorial Board for comments on the manuscript.

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