Simple Purification of Highly Active Biotinylated P-Glycoprotein: Enantiomer-Specific Modulation of Drug-Stimulated ATPase Activity†

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ABSTRACT: A simplified method for the expression and purification of P-glycoprotein (Pgp) is presented. This method is based on the in-frame fusion of both a polyhistidine tail and a 100-amino acid residue biotin acceptor domain of oxaloacetate decarboxylase from *Klebsiella pneumoniae* at the carboxyl terminus end of Pgp (Pgp-H6BD). The expression/purification protocol for Pgp-H6BD involves high-level expression of the fusion protein in the yeast *Pichia pastoris*, biotinylation in vitro with biotin ligase, solubilization of crude membrane fractions in detergent, and affinity purification by a combination of nickel and avidin chromatography. Biotinylated Pgp binds to immobilized monomeric avidin and can be eluted with free biotin in a high state of purity. This protocol is rapid and efficient and yields purified Pgp which shows robust ATPase activity, as determined by vanadate-induced trapping of photoactive nucleotides and by direct measurement of ATP hydrolysis by Pgp-H6BD. This method should be useful for structural studies of the protein by spectroscopic or crystallographic approaches. This purified Pgp-H6BD preparation has been used to study the enantiomer-specific effects of inhibitors of Pgp-mediated drug transport on the drug-stimulated ATPase activity of the protein. A series of 1,4-disubstituted piperazine derivatives with a central chiral carbon and modified at the head and tail groups are shown to stimulate Pgp ATPase activity in a dose-dependent fashion. Some of these compounds are also capable of inhibiting either vinblastine or verapamil stimulation of ATPase activity of Pgp in an enantiomer-specific fashion. The enantiomeric specific inhibitory activity of these compounds suggests complex interactions at a single substrate binding site(s) on Pgp.

P-glycoprotein (Pgp)[1] overexpression causes resistance to chemotherapeutic agents in tumor cells in vivo and cultured cells in vitro (*1*). Pgp is an integral membrane protein that functions as an ATP-dependent efflux pump to reduce intracellular drug accumulation in resistant cells (*2, 3*). Pgp binds [^{32}P]-8-azido-ATP, and purified Pgp shows robust ATPase activity (K_M for Mg^{2+}ATP of 1 mM, V_{max} of 0.3−2 μmol/min/mg of protein) (*2, 4*) that can be further stimulated by certain drug substrates [e.g. vinblastine (VBL)] or inhibitors of Pgp transport [e.g. verapamil (VRP)] (*3*). Biochemical studies in transfected mammalian cells (*5, 6*) or yeast cells (*7*), as well as genetic analyses of naturally occurring mutations in humans (*8*) or experimentally induced mutations in mice (*9*), have shown that at least one of the Pgp isoforms functions as a phospholipid translocator, possibly by a flippase mechanism (*7, 10*). It is believed that the mechanism of drug transport by Pgp in resistant cells is related to the mechanism of Pgp-mediated lipid transport in normal tissues.

Pgp is composed of 12 transmembrane (TM) domains and 2 nucleotide binding (NB) sites arranged in two symmetrical halves. This structural unit (6TM, 1NB) defines the ABC (ATP binding cassette) family of membrane transporters, which has members in bacteria, yeast, parasites, nematodes, plants, and mammals (*11*). In mammals, this family includes transporters for peptides (*12*), chloride ions (*13*), acylated fatty acids (*14, 15*), bile acids (*16*), and other substrates (*17*). Of particular interest is the multidrug-resistance-associated protein (MRP) family, composed of at least six members in humans (*18*), with MRP1 and MRP2 capable of active drug transport when overexpressed in transfected cells (*19, 20*). While Pgp can act on unmodified drug molecules, MRP1 and MRP2 can transport a variety of organic anions, including glutathione and/or glucuronide adducts (*21*).

Several mechanistic aspects of Pgp-mediated drug transport remain poorly understood. These include the apparent broad substrate specificity of the transporter, the mechanism of ATP binding and hydrolysis which occurs at both NB

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[1] Abbreviations: ADM, adriamycin; ABC, ATP-binding cassette; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BD, biotin domain; DM, *n*-dodecyl β-D-maltoside; DTT, dithiothreitol; Pgp, P-glycoprotein; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VBL, vinblastine; VRP, verapamil.

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sites, the mechanism by which drug binding to the protein induces ATP hydrolysis, and how ATP hydrolysis ultimately results in drug efflux. A better understanding of these problems may also shed light on the mechanism of transport by other ABC transporters, including MRP. Studies on mechanistic aspects of Pgp transport are best carried out with pure protein in a reconstituted system. In addition, structural studies on the protein require large amounts of highly purified protein. Over the years, several protocols have been developed for partial or complete purification of Pgp, either from drug-resistant cells expressing the protein endogenously or from model organisms expressing a recombinant protein. Early partial purification was achieved by immunoaffinity with a monoclonal anti-Pgp antibody (*22*). Anion exchange alone (*23*) or coupled with different types of affinity chromatography were also used, including substrate coupled to Sepharose (*24*), lectin resins (*25*), or dye−ligand agarose (*26*, *27*). An alternative approach utilizes short epitope tags in Pgp that can then be used as a bait for affinity chromatography. For example, a polyhistidine tail fused at the C-terminus of Pgp has been successfully used to obtain purified Pgp by nickel chelate chromatography from either mammalian (*28*), insect (*29*), or yeast (*30*) cells overexpressing the protein. Such preparations may still contain small amounts of contaminating proteins, possibly interfering with physical characterization of Pgp. A simpler, more rapid method that yields purified protein in a concentrated form would be useful for various applications.

Consler et al. (*31*) have fused in-frame a biotin acceptor domain (BD) from oxaloacetate decarboxylase of *Klebsiella pneumoniae* at the C-terminus of the lactose permease of *Escherichia coli*. A portion of the biotin domain is biotinylated in vivo (*31*), and the remainder could be biotinylated in vitro using purified biotin ligase (*32*) thereby increasing the yield. Biotin ligase catalyzes transfer of biotin from the intermediate, biotinyl-5′-AMP, to the unique lysine residue in the biotin domain (*33*, *34*). The strong affinity between biotin and avidin is used to obtain highly purified and biologically active biotinylated chimeric protein by chromatography on a monomeric avidin agarose resin.

In the current study, we have engineered a polyhistidine tail and a biotin acceptor domain at the C-terminus of the Pgp encoded by mouse *mdr3*. We describe a protocol for the rapid purification of this Pgp from membrane fractions of *Pichia pastoris* expressing large amounts of this protein. Pgp purified in such a fashion retains full ATPase activity and is used to study modulation of drug-stimulated ATPase activity by small molecule inhibitors of Pgp-mediated drug transport (*35*, *36*).

EXPERIMENTAL PROCEDURES

Construction of the mdr3-biotin Domain Chimera. Initially, a mutagenic oligonucleotide primer (3′-TACGTACATCACCATCACCATCACTCGAGCTGAGTCGAC-5′) was used to modify plasmid vector pBluescript KS. This primer introduces a polyhistidine tract and several new restriction sites (*Sna*BI, *Xho*I, *Sal*I; underlined) in the cloning polylinker of pBluescript, which had been altered to delete polylinker sites between *Sac*I and *Kpn*I. In a second step, the mutant variant of the biotin acceptor domain (BD) of oxaloacetate decarboxylase from *K. pneumoniae* with the single tryptophan residue replaced with phenylalanine (*32*) was inserted in-frame downstream of the 6-His tail, using the *Xho*I site. The mouse *mdr3* cDNA was modified at its 5′-end by mutagenesis to GAGCTCCCGCGGAAAAAAA**ATG** (initiator ATG bold, *Sac*I and *Sac*II sites underlined) to improve mRNA stability and protein expression in yeast as previously described (*37*). *mdr3* was then excised from plasmid pVT-*mdr3.5* (*30*) using *Kpn*I and *Sna*BI, and the ends were repaired with T4 DNA polymerase before cloning into the *Sna*BI site of the modified pBluescript vector to produce p*mdr3*-H6BD. For expression of the chimeric protein in the yeast *P. pastoris*, a 3.9-kb *Sac*I to *Sal*I *mdr3*-H6BD fragment was excised from plasmid p*mdr3*-H6BD, and its ends were repaired with T4 DNA polymerase before cloning into the *Eco*RI site of plasmid pHIL-D2 also repaired with T4 DNA polymerase to create pHIL-*mdr3*-H6BD. The integrity of the restriction sites used for cloning was verified by enzymatic digestion and the sequence of amplified cDNA subfragments used for subcloning was verified in the final construct. All oligonucleotide primers used for mutagenesis and DNA sequencing were obtained from GIBCO/BRL. Restriction enzymes, polymerases, and ligases used for cloning and sequencing were obtained from Pharmacia/Amersham and from New England Biolabs (Mississauga, Ontario).

Expression of the Chimeric mdr3-H6BD in P. pastoris. Transformation of pHIL-*mdr3*-H6BD in the methanotrophic yeast *P. pastoris*, selection of Mdr3-H6BD-producing recombinant clones, and preparation of enriched membrane fractions from *P. pastoris* were carried out as described by Urbatsch et al. (*30*) with the following modifications. Briefly, pHIL-*mdr3*-H6BD was transformed into *P. pastoris* strain GS115 according to the manufacturer's instructions (InVitrogen, license number 145 457) using a lithium chloride technique. His^+ transformants showing successful homologous recombination at the *AOX1* locus were identified as unable to grow on medium containing methanol (*m*ethanol *u*tilizing slow or mut^s). Over 90% of the His^+mut^s transformants identified were found to express the chimeric Mdr3-H6BD protein, as determined by immunoblotting of membrane fractions with the mouse anti-Pgp monoclonal antibody C219 (Signet, Toronto, Ontario) (*30*). For large-scale preparations, cultures were induced in methanol-containing media for 48 h, and crude plasma membranes were isolated by centrifugation after cell lysis using a French press (*37*). From a 2-L culture, between 100 and 150 mg of crude membrane protein was obtained routinely. The crude membranes were resuspended in 50 mM Tris-HCl, pH 8.0, 20% glycerol, and 50 mM NaCl with freshly prepared protease inhibitors (1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin A; Boehringer) and stored at −80 °C until use.

In Vitro Biotinylation. Crude membranes (approximately 100 mg) were thawed on ice and resuspended to a final volume of 3 mL of 40 mM Tris, pH 7.4, 0.1 mM EGTA. Biotinylation was carried out directly on the crude membrane fraction in vitro using a commercially available set of reagents and a modification of an experimental protocol from Avidity Co. (*38*). Briefly, crude membrane extracts were supplemented with 350 μL of 0.5 M Bicine buffer (pH 8.3) and 350 μL of a solution containing 100 mM ATP, 100 mM Mg-O-acetate, and 400 μM D-biotin in the presence or absence of biotin ligase (12 500 U). The mixture was then incubated at 37 °C for 3 h, with occasional shaking. The

extent of biotinylation of Mdr3-H6BD was monitored by immunoblotting with extravidin peroxidase (Sigma, St. Louis, MO) followed by detection of specific complexes with the ECL detection system (Amersham).

Solubilization and Nickel Chelate Chromatography. The conditions for solubilization of the Mdr3-H6BD protein were a modification of a protocol previously described (*30*). Briefly, 50 mg of crude membrane proteins was precipitated with 10 mM $MgCl_2$ (30 min at 4 °C) to remove EGTA and free d-biotin and recovered by centrifugation (16000*g*, 20 min). Membrane pellets were resuspended in 50 mM Tris, pH 8, 20% glycerol, 50 mM NaCl, 5 mM imidazole, 0.5 mM β-mercaptoethanol (buffer A) and solubilized by adding an equal volume of 0.6% L-α-lysophosphatidylcholine (lyso-PC, from egg yolk, 99% pure; Sigma) in buffer A at a protein concentration of 2 mg/mL, followed by gently vortexing at 20 °C. Samples were next centrifuged at 60000*g* for 30 min at 4 °C to remove particulate material. Solubilized proteins were mixed with 700 μL of packed Ni-NTA resin (Qiagen), equilibrated with buffer A, followed by incubation at 4 °C for 20 h with continuous rotation. The resin was transferred to a column and washed extensively with 20 bed volumes of 50 mM Tris, pH 8, 20% glycerol, 50 mM NaCl, 5 mM imidazole, 0.5 mM β-mercaptoethanol, 0.1% *n*-dodecyl β-D-maltoside (DM) (buffer B) followed by an additional 20 bed volumes of buffer B containing 15 mM imidazole. Proteins bound to the Ni-NTA resin were eluted with 3 mL of a buffer containing 50 mM Tris-Cl, pH 8.0, 20% glycerol, 80 mM imidazole, 0.1% DM. All buffers used in the purification procedures contained freshly prepared protease inhibitors (1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin A; Boehringer).

Avidin Affinity Chromatography. The Mdr3-H6BD protein was purified on monovalent avidin-Sepharose by a modification of a protocol developed for the purification of biotinylated lactose permease (*32*). Briefly, 0.5 mL of packed monovalent avidin resin (Pierce) was washed sequentially with 10 volumes of buffer B, 10 volumes of buffer B supplemented with 4 mM D-biotin, 10 volumes of 0.1 M glycine, pH 2.8, and finally 10 volumes of buffer B again. The 3-mL eluate from the nickel column was incubated overnight at 4 °C with washed avidin resin with continuous rotation. The resin was transferred into a column and washed extensively with 20 bed volumes of buffer B followed with 20 bed volumes of buffer B containing 100 nM of D-biotin to remove nonspecifically bound proteins. Bound Mdr3-H6BD was eluted with 3 mL of 4 mM D-biotin in buffer B. For reconstitution, the purified protein was incubated with 1% *E. coli* lipids (acetone/ether preparation) and 1 mM DTT for 30 min on ice, followed by dialysis (16 h, 4 °C) against a buffer containing 50 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, and 1 mM DTT. The reconstituted protein was concentrated by centrifugation (200000*g*, 2 h, 4 °C) and was resuspended in a final volume of 300 μL of dialysis buffer. This final suspension was aliquoted and stored at −80 °C until use.

Assay of ATPase Activity. For ATPase assays, 0.4 μg of dialyzed Mdr3-H6BD was added to a reaction mixture consisting of 50 mM Tris-Cl, pH 8.0, 0.1 mM EGTA, 10 mM Na_2ATP, and 10 mM $MgCl_2$ in a final volume of 200 μL and incubated at 37 °C; 50-μL aliquots of this mixture were removed at the appropriate times during which the reaction was linear and less than 10% of the added nucleotide was hydrolyzed. Reactions were stopped by addition of 1 mL of 20 mM ice-cold H_2SO_4. P_i release was assayed by the method of Van Veldhoven and Mannaerst (*39*). For determination of kinetic parameters, an excess of 2 mM $MgCl_2$ over MgATP concentrations was used. Drugs and modulators were added at various concentrations from dimethyl sulfoxide stock solutions (1 mg/mL) and the final solvent concentration in the assay was kept at ≤2% (v/v).

Vanadate Trapping of Mg-8-azido[α-^{32}P]ATP in Mdr3-H6BD. Dialyzed Mdr3-H6BD was incubated with 5 μM 8-azido[α-^{32}P]ATP (8.5 Ci/mmol, ICN), 3 mM $MgCl_2$, 50 mM Tris-Cl, pH 8.0, 0.1 mM EGTA in a total volume of 50 μL at 37 °C for indicated times. VRP (100 μM) and vanadate (200 μM) were included where indicated. Incubations were started by addition of 8-azido[α-^{32}P]ATP and stopped by transfer to ice. Free label was promptly removed by centrifugation at 200000*g* for 30 min at 4 °C in a TL-100 rotor (Beckman), and the proteoliposomes were washed and resuspended in 50 μL of ice-cold 50 mM Tris-Cl, pH 8.0, 0.1 mM EGTA. Samples were kept on ice and irradiated with UV light for 5 min (UVS-II Mineral light 260 nm placed directly above the samples). At concentrations of 5 μM 8-azido[α-^{32}P]ATP, hydrolysis and subsequent vanadate-induced trapping of nucleotide is about 20 times slower than at 80 μM 8-azido[α-^{32}P]ATP, a concentration previously used in vanadate-trapping experiments (*30*). Upon UV irradiation, up to 10% of mouse Mdr3 is covalently labeled by 8-azido-[α-^{32}P]nucleotide (*30*). Samples were dissolved in 5% (w/v) SDS, 25% (v/v) glycerol, 0.125 M Tris-Cl, pH 6.8, 40 mM DTT, 0.01% pyronin Y for 30 min at 37 °C, separated on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The membrane was exposed overnight at −80 °C to Kodak BioMax films with intensifying screens. Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 (Fisher Scientific) at pH 10 as described (*40*) and boiled for 2 min before each use to break down polymeric species.

Effect of Inhibitors on Pgp-Mediated Drug Transport. The inhibitory potential of synthetic compounds for Pgp activity was measured in dose−response experiments by their ability to block Pgp-mediated transport of [^{14}C]adriamycin (ADM). The Pgp-positive multidrug-resistant human carcinoma cell line KBV1 was obtained from Dr. M. M. Gottesman (NIH, Bethesda, MD). The cell line was routinely maintained in Dulbecco's modified Eagle Medium (DMEM) containing 1μg/mL VBL and supplemented with 10% fetal calf serum, penicillin (50 units/mL), streptomycin (100 μg/mL), and gentamycin (25 μg/mL). For drug transport assays, 24 h prior to experimentation, KBV1 cells were seeded in 6-well plates (1.2×10^6 cells/well) in DMEM lacking VBL but supplemented with 5% fetal calf serum and antibiotics. To measure ADM accumulation, cells were exposed for 3 h to [^{14}C]ADM (specific activity of 47.3 μCi/mmol; final concentration 2 μM) in the absence or presence of increasing concentrations of modulators. Cells were washed twice with ice-cold PBS and collected by trypsinization. Cell number and viability were determined and cell-associated radioactivity was measured by suspending collected cells in liquid scintillation fluid (Beckman, Ready Safe), vortexing, and scintillation counting. The potencies of the compounds to modulate Pgp function were determined by calculating the concentration of compound which increases basal accumulation (determined in inhibitor free conditions) of ADM in KBV1 cells by 300%.

Routine Procedures. Protein concentrations were determined by the bicinchoninic acid method (Pierce) in the presence of 1% SDS using bovine serum albumin as a standard. SDS−PAGE was performed according to Laemmli (*41*) using the Mini-PROTEAN II gel and Electrotransfer system (BioRad). Samples were dissolved in 5% (w/v) SDS, 25% (v/v) glycerol, 0.125 M Tris-Cl, pH 6.8, 40 mM DTT, 0.01% pyronin Y for 30 min at 37 °C and separated on 7.5% polyacrylamide gels. For immunodetection of Pgp, the mouse monoclonal antibody C219 (Signet Laboratories Inc.) was used with the ECL detection system (Amersham).

Materials. Biotinylation kits were purchased from Avidity. Monovalent avidin-Sepharose was from Pierce. 8-Azido[α-^{32}P]ATP, VRP, and VBL were purchased from ICN. All other compounds were synthesized at Pfizer Central Research, Groton, CT. They are enantiomers of the 1,4-disubstituted piperazine analogues (see Figure 6) containing either a quinoline (CP162398, CP162399) or a benzothiazole (CP172732, CP219994) tail and a benzhydryl derivative as the headgroup or a quinoline tail and a tricyclic stucture as the headgroup (CP147673, CP147674). The *R*(+) and the *S*(−) enantiomers were purified to optical purities >95% excess. CP162398/399 and the racemate of CP147673/674 have been previously described in the literature (*35, 36*). Acetone/ether-precipitated *E. coli* lipids were from Avanti Polar Lipids, and general chemicals were of reagent grade from Sigma.

RESULTS

Expression and in Vitro Biotinylation of Mdr3-H6BD. The biotin acceptor domain of oxaloacetate decarboxylase from *K. pneumoniae* with a short segment coding for six consecutive histidine residues was fused in-frame at the carboxyl terminus end of Mdr3 to generate Mdr3-H6BD. The pHIL*mdr3-H6BD* construct was introduced at the chromosomal *AOX1* locus of the methanotropic yeast *P. pastoris* by homologous recombination, as we have previously described (*37*). Independent *P. pastoris* transformants showing loss of *AOX1* function were screened for Mdr3-H6BD protein expression in response to methanol induction, by Western blotting using the mouse anti-Pgp monoclonal antibody C219 (*30*). Several positive clones were identified, and one of them showing good expression level was retained for further analysis (data not shown). As a first step, we wanted to verify that the biotin acceptor domain used in our construct could indeed be biotinylated in vitro after expression in *P. pastoris*. Briefly, membrane fractions from the Mdr3-H6BD-expressing clone were incubated with biotin in the presence or absence of added biotin ligase, followed by SDS−PAGE, transfer to nitrocellulose membrane, and development with extravidin peroxidase (Figure 1A). In absence of biotin ligase (lane 1), Mdr3-H6BD labeling was readily detected. This labeling was specific, was not seen in cell extracts from control cells (data not shown), and probably results from endogenous biotin ligase activity present in *P. pastoris* extracts. Increasing the D-biotin concentration in the methanol-containing induction medium did not enhance this endogenous activity, resulting in the same amount of biotinylated Pgp as control conditions (data not shown). The addition of external biotin ligase to the reaction mixture causes at least a 15−20-fold increase in the amount of biotinylated Mdr3-H6BD (Figure 1A, lane 2), compared to the control (lane 1). This increase in biotinylation is specific and does not result in increase biotinylation of other *P. pastoris* proteins. Finally, Western blotting experiments with anti-Pgp antibody C219 (Figure 1B) show that both biotinylation reactions contain the same amount of immunoreactive Mdr3-H6BD. Together, the results show that Mdr3-H6BD expressed in membranes from *P. pastoris* can be efficiently biotinylated in vitro by added biotin ligase.

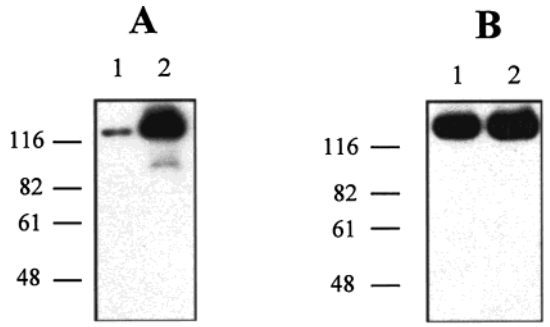

FIGURE 1: Biotinylation of Mdr3-H6BD in vitro. Crude membrane fractions from *P. pastoris* yeast cells expressing the Mdr3-H6BD protein were incubated (3 h at 37 °C) with a biotinylation reaction mixture in the absence (lane 1) or presence (lane 2) of exogenously added biotin ligase. Samples were separated on 7.5% polyacrylamide SDS gels, transferred to nitrocellulose membranes, and analyzed by Western blot using extravidin peroxidase (A). The membrane was stripped and analyzed by immunoblotting for the presence of immunoreactive Pgp, using the mouse monoclonal anti-Pgp antibody C219 (B).

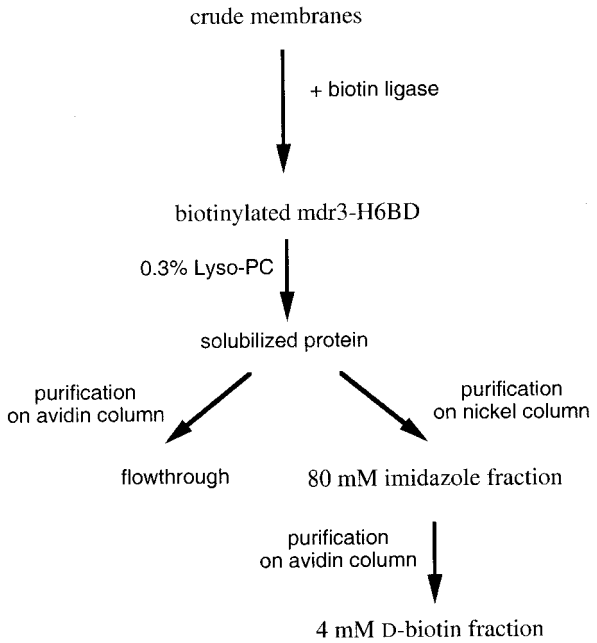

FIGURE 2: Purification protocol for the Mdr3-H6BD protein.

Purification of the Mdr3-H6BD Protein. To assess the efficiency of purification of the biotinylated Mdr3-H6BD protein by avidin chromatography, parallel experiments were carried out in which the purification of in vitro biotinylated (Figure 3A) vs nonbiotinylated (Figure 4A) protein was compared. Various column fractions and eluates corresponding to each purification step were analyzed by SDS−PAGE, and the identity of the major Pgp species detected by Coomassie blue staining was verified by immunoblotting using the mouse monoclonal anti-Pgp antibody C219 (Figures 3B and 4B).The experimental procedure for avidin

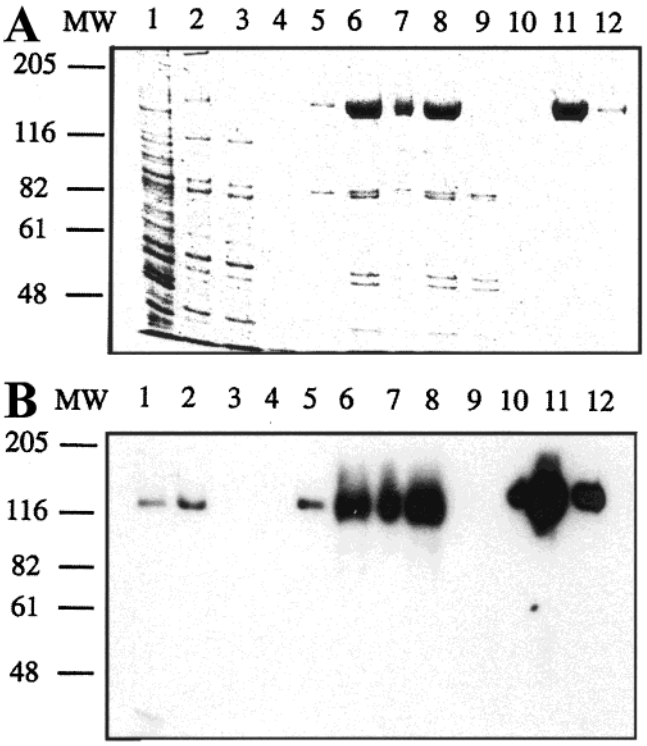

FIGURE 3: Purification of Mdr3-H6BD biotinylated in vitro using biotin ligase. Crude membrane fractions prepared from *P. pastoris* expressing Mdr3-H6BD were biotinylated in vitro, solubilized in 0.3% lysophosphatidylcholine (lyso-PC), and purified on Ni-NTA resin and by avidin affinity chromatography as described under Experimental Procedures. Samples from each purification step were separated on 7.5% polyacrylamide SDS gels and stained with Coomassie blue (A) or transferred to a nitrocellulose membrane and analyzed by immunoblotting with the mouse monoclonal anti-Pgp antibody C219 (B). Every sample contained 25 μg of protein, except for the 10% SDS wash of the resin. Purification on the Ni-NTA column is shown in lanes 1−7 and includes aliquots of microsomal membranes after in vitro biotinylation (lane 1), extract loaded on the Ni-NTA column (lane 2), pools of flowthrough from the Ni-NTA column (lane 3), wash material obtained with buffer containing imidazole at 5 mM (lane 4) and 15 mM (lane 5), the 80 mM imidazole eluate (lane 6), and a 10% SDS wash (lane 7). The avidin affinity chromatography is described in lanes 8−12 and includes aliquots of Mdr3-H6BD loaded on the avidin resin (lane 8), flowthrough from the chromatography (lane 9), wash material obtained with buffer containing 100 nM D-biotin (lane 10), the 10 mM D-biotin eluate (lane 11), and a 10% SDS wash (lane 12). The position of the molecular mass markers is given in kDa.

affinity chromatography of Mdr3-H6BD was carried out essentially as described by Pouny et al. (*32*), with one important modification (see schematic representation in Figure 2). In pilot experiments using an extensive washing procedure to remove unreacted biotin after in vitro biotinylation, we noted after detergent solubilization and incubation with avidin (overnight at 4 °C) that most of the protein was found in the flowthrough of the column (data not shown). This was interpreted as presence of residual biotin in the protein sample possibly preventing efficient binding of the biotinylated protein to the avidin resin. To circumvent this problem, we took advantage of the polyhistidine tail engineered in-frame with the BD in Mdr3-H6BD, and that allows partial purification of the protein by nickel chelate chromatography (Figures 3 and 4). Thus, in the final purification protocol, a crude membrane extract from Mdr3-H6BD *P. pastoris* cells was biotinylated in vitro (lane 1), followed by

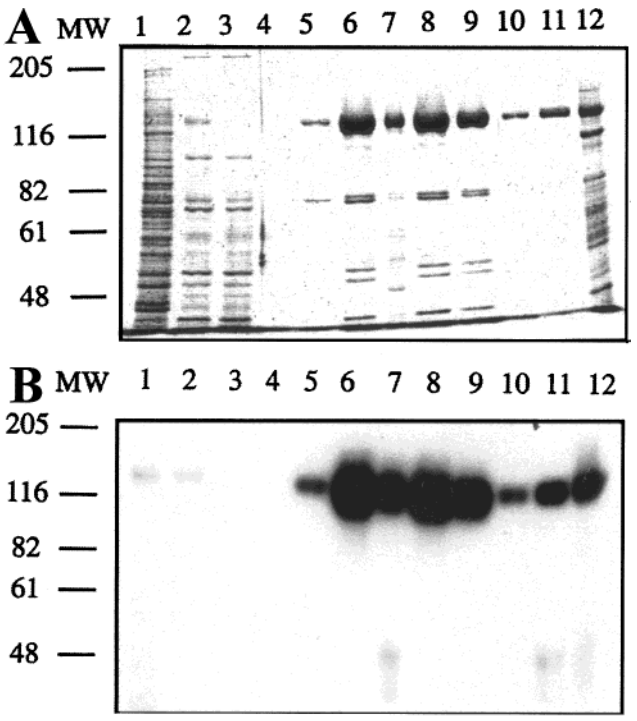

FIGURE 4: Purification of Mdr3-H6BD biotinylated in vitro in the absence of biotin ligase. The legend is identical to that for Figure 3, except that the Mdr3-H6BD biotinylation reaction was carried out in the absence of exogenously added biotin ligase.

solubilization in a buffer containing 0.3% lysophosphatidylcholine (lyso-PC; lane 2) and elimination of insoluble material by centrifugation, before overnight incubation with Ni-NTA resin (Quiagen). This material was then chromatographed, and the flowthrough (lane 3), together with serial washes of the column in a buffer containing 5 mM (lane 4) and 15 mM imidazole (lane 5), as well as the final eluate in 80 mM imidazole (lane 6) and a 10% SDS wash (lane 7) were analyzed by gel electrophoresis. Under these conditions, we noted that all Mdr3-H6BD that bound the Ni-NTA resin (compare lanes 2 and 3) the most could be eluted at 80 mM imidazole (lane 6), with only a residual amount trapped on the column (lane 7). We also noted a small amount of non-Mdr3-H6BD material co-purifying with the protein in the 80 mM imidazole eluate and/or remaining irreversibly bound to the column and detected in the 10% SDS fraction, as previously described (*30*). The Ni^{2+} chelate purification procedure appeared as efficient for the biotinylated and nonbiotinylated proteins (compare Figures 3A and 4A). The 80 mM imidazole fraction (lane 8) was then incubated with the avidin resin for different periods of time. In preliminary experiments, we noted that a 4-h incubation period at 4 °C was insufficient to achieve complete binding of Mdr3-H6BD (data not shown). On the other hand, after overnight incubation at 4 °C with continuous, gentle agitation, all biotinylated Mdr3-H6BD was bound to the resin and remain bound in 100 nM biotin [compare lanes 9 and 10 for biotinylated (Figure 3A) vs nonbiotinylated (Figure 4A) Mdr3-H6BD]. Bound Mdr3-H6BD could be eluted efficiently with 4 mM D-biotin (Figure 3A, lane 11), while only a small amount of Mdr3-H6BD as well as other contaminants remained irreversibly bound to the resin (lane 12, SDS wash). In this analysis, we noted that the purity of the Mdr3-H6BD was greater than that seen with Ni-NTA column chroma-

tography and was seemingly free of contaminating material even in the very low molecular mass range. A parallel purification of Mdr3-H6BD, where biotin ligase had been omitted during the in vitro biotinylation step (Figure 4A,B), shows that a large proportion of Mdr3-H6BD did not bind to the avidin resin and the amount of Pgp eluted with 10 mM biotin corresponded to the amount of biotinylated protein in vivo (see Figure 1). In all cases, the major Mdr3-H6BD band detected by Coomassie blue was C219-immunoreactive Pgp (Figures 3B and 4B). Using this purification method with 100 mg of crude microsomal membrane proteins from Mdr3-H6BD *P. pastoris*, a yield of 700 μg of purified protein was obtained routinely.

ATPase Activity of the Purified Mdr3-H6BD Protein. Wild-type Pgp shows a strong, drug-stimulated ATPase activity with measured K_M of 0.7−0.9 mM and V_{max} of 3−4.3 μmol/min/mg of protein for Pgp purified from *P. pastoris* (*30*). Two methods were used to assess functional integrity of the purified Mdr3-H6BD with respect to ATPase activity: phosphate release in solution and vanadate-induced trapping of Mg^{2+}-8-azido[α-^{32}P]ATP, as we have previously described (*30*).

It has been previously established that vanadate trapping of Mg^{2+}-8-azido[α-^{32}P]ATP by Pgp requires hydrolysis of ATP and that the trapped nucleotide species is ADP (*42*). Thus, this technique can be used to rapidly evaluate the ATPase activity of Pgp. Purified and dialyzed Mdr3-H6BD was preincubated for 15 min at 37 °C with 5 μM 8-azido-[α-^{32}P]ATP, 3 mM $MgCl_2$ in the absence or presence of 100 μM VRP and 200 μM vanadate. Unbound ligands were removed by ultracentrifugation followed by UV irradiation and analysis by electrophoresis on SDS gel. The gel was blotted to a membrane for autoradiography (Figure 5B) and was also analyzed for equal protein loading by immunoblotting with monoclonal antibody C219 and ECL detection (Figure 5A). Vanadate-induced photolabeling by the radionucleotide is readily detectable in the Mdr3-H6BD protein in the absence of drug (Figure 5B, lane 1). Labeling is significantly enhanced by the addition of VRP (100 μM; Figure 5B, lane 3) but is completely eliminated if vanadate is omitted from the reaction (Figure 5B, lane 2). Immunoblotting shows that equal amounts of Pgp were loaded in each lane of the gel (Figure 5A). VRP-stimulated ATPase activity is readily detectable in the purified Mdr3-H6BD fractions, with a measured V_{max} of 2.4 μmol/min/mg of protein in the presence of 100 μM VRP (Figure 5C). This corresponds to a 10-fold stimulation above the low background ATPase activity measured in the absence of stimulator (0.25 μmol/min/mg of protein; Figure 5C). VBL also stimulates ATP hydrolysis by Mdr3-H6BD by a factor of 3 (0.81 μmol/min/mg of protein; Figure 5C). The maximum drug-stimulated ATPase activity of the purified Mdr3-H6BD protein is very similar to that of other Pgp preparations purified from mammalian (*43, 44*) and yeast (*30*) cells. For both stimulators, the K_M for ATP is 0.68−0.75 mM (Figure 5C), also in good agreement with previously reported K_M values for Pgp. This indicates that the Mdr3-H6BD purified from *P. pastoris* by the described procedure is fully functional and that the hexahistidine tag and biotin acceptor domain do not alter function.

Modulation of the Drug-Stimulatable ATPase Activity of Mdr3-H6BD. We used the purified Mdr3-H6BD protein to explore the relationship between the inhibitory properties of certain small molecule inhibitors of Pgp and their possible effect on the basal and drug-induced ATPase activity of Pgp. We have previously described the activity of a series of synthetic compounds in reversing VBL resistance of CHO cells transfected with wild-type mouse and human Pgps (*35, 36*). One of these compounds is a 1,4-disubstituted piperazine analogue that possesses a central chiral carbon atom (Figure 6). In the current study, we have analyzed the anti-Pgp activity of three structurally related enantiomer pairs of such

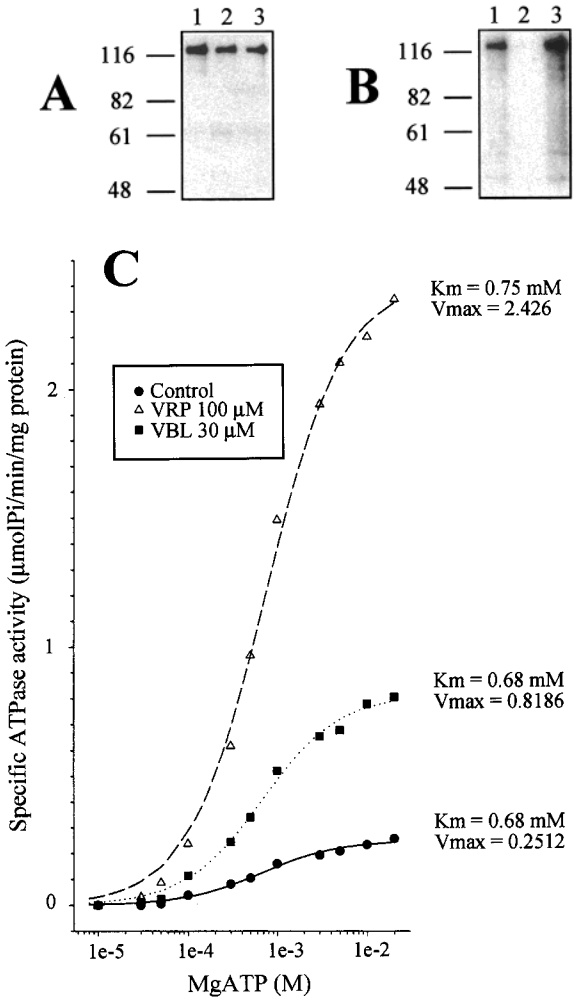

FIGURE 5: ATPase activity of the purified Mdr3-H6BD protein. Vanadate-induced trapping of 8-azido[α-^{32}P]ATP in purified Mdr3-H6BD (B). Purified, dialyzed Mdr3-H6BD was preincubated with 5 μM 8-azido[α-^{32}P]ATP, 3 mM $MgCl_2$ in the absence or presence of 100 μM VRP and 200 μM vanadate at 37 °C: lane 1, 200 μM vanadate; lane 2, control without vanadate or VRP; lane 3, 100 μM VRP and 200 μM vanadate. Unbound ligand was removed by ultracentrifugation followed by UV irradiation (*30*). Photolabeled samples were separated by electrophoresis on polyacrylamide SDS gels, followed by transfer to nitrocellulose membrane and autoradiography. (A) The membrane was then analyzed for Pgp expression by immunoblotting using the mouse monoclonal anti-Pgp antibody C219. (C) The specific ATPase activity of purified Mdr3-H6BD was measured as a function of Mg^{2+}ATP concentrations in the absence or presence of 100 μM VRP or 30 μM VBL. The raw data of a single representative experiment where every data point corresponds to a duplicate measurement are shown. The maximum velocity in the presence of VRP was 2.4 μmol/min/mg of protein, in the presence of VBL 0.81 μmol/min/mg of protein, and in the absence of drug 0.25 μmol/min/mg of protein. The curves are nonlinear least-squares regression fits of the data points to the Michaelis−Menten equation using Sigmaplot program.

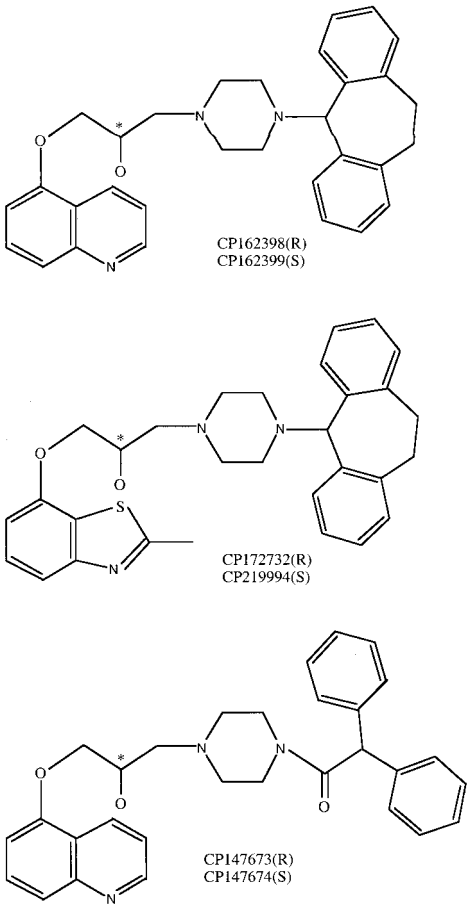

FIGURE 6: Molecular structures of the 1,4-disubstituted piperazine analogue Pgp inhibitors. The position of the asymmetric chiral carbon atom is indicated (*).

Table 1: Modulatory Activity of 1,4-Disubstituted Piperazine Analogues on ADM Transport by Pgp

modulator	enantiomer	Pgp inhibition (EC3X) (μM)[a]
CP162398	*R*	0.05
CP162399	*S*	0.13
CP147673	*R*	0.62
CP147674	*S*	1.2
CP172732	*R*	0.06
CP219994	*S*	0.11

[a] Corresponds to the modulator concentration necessary to increase [^{14}C]ADM accumulation in Pgp-overexpressing cells by a factor of 3-fold (EC3X).

1,4-disubstituted piperazine analogues. The first pair (CP162398,CP162399) has a quinoline at position 4 and a tricyclic structure at position 1. In the second pair (CP172732, CP219994), the quinoline group is substituted by a benzothiazole moiety, and in the third pair, the tricyclic group is replaced by a benzhydryl derivative at position N-1 (CP147673,CP147674). The *R* and *S* enantiomers of each compound were tested for their anti-Pgp activity, by monitoring in dose−response experiments their ability to increase by a factor of 300% (EC3X) the accumulation of [^{14}C]ADM in Pgp-positive KBV1 drug-resistant cells. Results summarized in Table 1 show that all six compounds have robust anti-Pgp inhibitory property with EC3X values ranging from the nanomolar range (CP162398, 50 nM) to the low micromolar range (CP147674, 1.2 μM). The *R* isomer is in all cases 2−3-fold more potent than the *S* isomer.

Using the purified Mdr3-H6BD protein, we investigated further the mechanistic basis of inhibition of Pgp-mediated drug transport by these compounds; in particular their ability to modulate either the basal or the drug-stimulated ATPase activity of Mdr3-H6BD was tested (Figure 7). In dose−response experiments, all six compounds behave very similarly and stimulate ATP hydrolysis by Mdr3-H6BD (Figure 7A). Stimulation is dose-dependent and maximal at about 3 μM where 4-fold stimulation (1 μmol/min/mg of protein) over basal activity (0.23 μmol/min/mg of protein) is observed for five compounds, while 10-fold stimulation of ATPase activity is seen for CP147674. Thus, these six compounds, like VRP, block Pgp transport but stimulate its ATPase activity.

We also tested whether these compounds could modulate the ATPase activity of Mdr3-H6BD stimulated by either VRP or VBL. In these experiments the concentration of the stimulator was kept fixed while the concentration of the modulators was varied. Under maximum stimulation conditions by VRP (100 μM; V_{max} 2.15 μmol/min/mg of protein, 8−9-fold stimulation over basal activity), five of the six compounds behaved in a very similar fashion and caused a dose-dependent inhibition of the VRP-stimulatable ATPase activity (Figure 7B). Inhibition is maximal at 3 μM (50% inhibition of activity) and is sometimes reduced at higher concentrations of the five compounds (e.g. 10 μM). Maximum inhibition of VRP-stimulated Mdr3-H6BD ATPase activity occurs at a concentration (3 μM) which is also optimal for stimulation of the basal ATPase activity of the protein by the five compounds (Figure 7A). One notable exception is CP147674 that has no effect on VRP-stimulated ATPase activity. Thus, since CP147674 differs from the other analogues by substitution of the tricyclic headgroup to a relatively less rigid and less lipophilic biphenyl moiety, this suggests that the headgroup may be important for competitive interactions with VRP. However, results from *R* enantiomer CP147673 indicate that this interaction is enantiomer-specific.

The modulator effect of the six analogues on VBL-stimulated ATPase activity of Mdr3-H6BD was tested and found to be distinct in several aspects from that seen for VRP-stimulated ATPase. Under conditions of maximum stimulation by VBL (30 μM; V_{max} 0.75 μmol/min/mg of protein, 3-fold stimulation over basal activity), the benzothiazoles, CP172732 and CP219994, had no effect on VBL-stimulated ATPase activity. The *S* enantiomers of the two quinolines (CP162399, CP147674) but not the corresponding *R* enantiomers (CP162398, CP147673) showed a robust inhibition of VBL-stimulated ATPase activity, with a maximum inhibitory dose of 3 μM which is also optimal for stimulation of basal ATPase activity of the protein by the three compounds. The absence of inhibition of VBL-stimulated ATP hydrolysis by benzothiazoles, CP172732 and CP219994, suggests that the quinoline tail is important for inhibition of VBL-mediated stimulation of ATPase activity of Pgp. On the other hand, substitution of the tricyclic ring headgroup of CP162399 with a biphenyl moiety in CP147674 has no effect on inhibition of VBL-stimulated ATPase activity. Finally, the inhibitory effect of the two active compounds is strictly enantiomer-specific, for the *S* enantiomer.

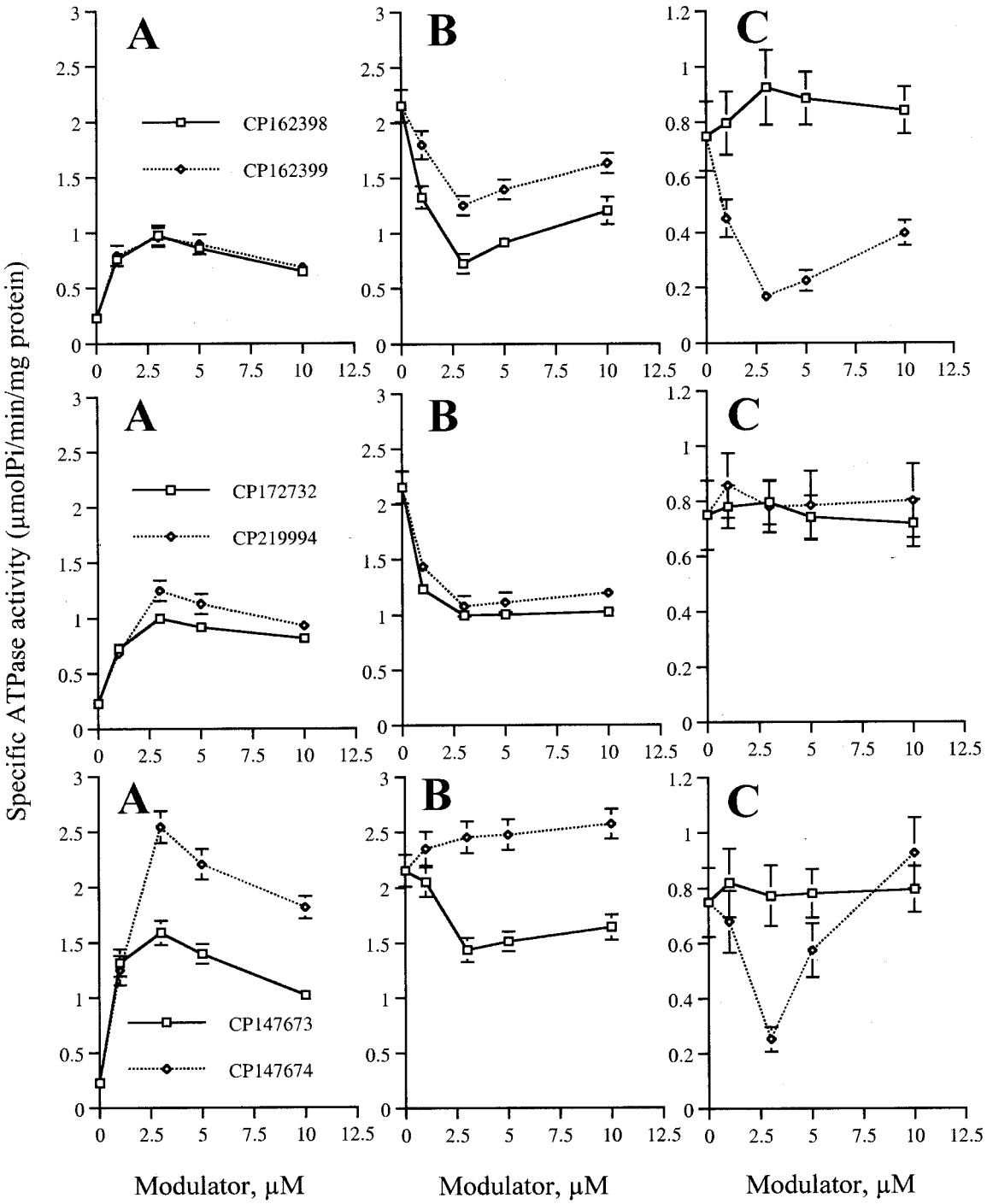

FIGURE 7: Modulation of basal and drug-stimulated ATPase activity of Mdr3-H6BD by disubstituted piperazine analogue Pgp inhibitors. The modulatory activity of 1,4-disubstituted piperazine enantiomer pairs (see structures in Figure 6), CP162398(*R*)/CP162399(*S*) (top three graphs), CP172732(*R*)/CP219994(*S*) (middle three graphs), and CP147673(*R*)/CP147674(*S*) (bottom three graphs), on basal or substrate-induced ATPase activity of Pgp was tested. The effect of the six compounds on the basal ATPase activity of Mdr3-H6BD was measured in dose−response experiments (A). The effect of the six compounds on the drug-stimulated ATPase activity of Mdr3-H6BD was also measured in dose−response experiments in the presence of VRP (100 μM) (B) or VBL (30 μM) (C). In these assays, the effect of the various modulators on the specific ATPase activity was measured using purified, dialyzed Mdr3-H6BD. Each point shows the mean ± standard deviation of triplicate experiments.

DISCUSSION

In this report, we present a novel method for rapid and efficient isolation of high-purity recombinant Pgp (mouse Mdr3) modified by the addition of a hexahistidine tail and biotin acceptor domain fused in-frame at its C-terminus. The method relies on several key experimental steps: (a) expression of sufficiently high amounts of Pgp in the membrane fraction of the methanotropic yeast *P. pastoris*, (b) efficient biotinylation in vitro with recombinant biotin ligase and solubilization of the protein in detergent, (c) elimination of the excess D-biotin by Ni-NTA chromatography, and (d) final affinity purification by avidin chromatography. Analysis of the purified material by SDS−PAGE and Coomassie blue staining identifies a single protein species, which is essentially free of contaminating material. In our hands, this protocol leads to a Pgp product of higher purity than that

obtained by protocols based on Ni-NTA purification alone (*30*). The avidin affinity chromatography step seems particularly efficient as very little material either is lost in the flowthrough or remains attached to the column after D-biotin elution. Under the experimental conditions described, the purified Mdr3-H6BD is highly functional as judged by drug-stimulated ATPase activity characteristic of this protein monitored by both vanadate-induced trapping of 8-azidoATP and ATP hydrolysis using a P_i release method (Figure 5). Under conditions of maximum stimulation by VRP and VBL, the measured specific activities were 2.4 and 0.81 μmol of P_i released/min/mg of protein, respectively, with a K_M for Mg^{2+}ATP of 0.68−0.75 mM. These values are equal or superior to those measured for wild-type Pgp expressed in the membrane of mammalian cells or for Pgp purified from such membranes or from membranes of yeast cells by different methods (*3, 4, 30, 43, 44*).

This avidin affinity purification procedure should be useful to obtain large amounts of pure wild-type or mutant variants of Pgp that may be required for detailed biochemical, biophysical, and structural studies of the protein. In particular, spectroscopic analysis of the protein by techniques such as tryptophan fluorescence and site-specific modifications by sulfhydryl reagents in single-cysteine mutants (*45*), as well as high-resolution structural data by electron microscopy or X-ray crystallography, all require highly pure protein that can be obtained by this method. Finally, the method can be adapted for purification, reconstitution, and biochemical characterization of other mammalian ABC transporters such as MRP (*46*) and CFTR (*47*) that have so far proven difficult to purify in an active form in sufficient amounts. We are currently adapting the protocol for purification of a member of the MRP family (Cie and Gros, unpublished).

We have used highly purified Mdr3-H6BD to investigate further the mechanism of action of a particularly potent group of Pgp inhibitors that have been previously characterized in intact cells (*35, 36*). This group of molecules was initially identified by the study of compound CP117227 which is a highly hydrophobic synthetic 1,4-disubstituted piperazine derivative with a chiral hydroxyl group at its asymmetric center and bearing a quinoline ring at position N-4 and a tricyclic structure at position N-1 (Figure 6). The racemic mixture (CP117227) and that of the *R* (CP162398) and *S* (CP162399) enantiomers are specific and potent inhibitors (nanomolar range) of multidrug resistance mediated by the human (MDR1) and murine (Mdr1, Mdr3) Pgps, as measured by ability to reverse cellular VBL resistance in corresponding CHO transfectants (*36*). Furthermore, in [^{3}H]VBL transport experiments, both the *R* and *S* enantiomers can block VBL transport by Pgp when used in the low micromolar range, with the *R* enantiomer showing superior activity in this assay (by a factor of 10-fold). Studies with radiolabeled analogues of CP162398 and CP162399 show that neither compound is transported by Pgp (*35*). This suggests that their inhibitory mode of action is either through a noncompetitive interaction at a low-affinity drug binding site shared with VBL or by allosteric inhibition at an unrelated site on the Pgp molecule. In the present report, additional analogues were synthesized for structure−activity relationship studies in this molecule, in which the quinoline ring was substituted by a benzothiazole (CP172732/CP219994) or in which the tricyclic headgroup was substituted by a benzhydryl structure (CP147673/CP147674). The anti-Pgp activity of these new compounds was tested by monitoring their ability to block ADM transport in Pgp-positive KB-V1 carcinoma cells. Although, there were differences in the EC3X values determined for these compounds, all four new compounds showed strong inhibitory activity in the low micromolar to nanomolar range.

The molecular basis of Pgp inhibition by these compounds was then investigated further by evaluating their effect on basal and drug-stimulated ATPase activity. As described for many Pgp substrates (e.g. VBL) and inhibitors (e.g. VRP) of transport, these six compounds strongly stimulate the basal ATPase activity of Pgp. Two of these, CP162398 and CP162399, have been previously shown to not be substrates for Pgp-mediated drug transport. Thus, these six compounds may bind to Pgp, stabilizing an ATPase-activated conformation that is no longer capable of drug transport. Further analysis of the effect of the three compounds on VRP- and VBL-stimulated ATPase activity suggests a complex relationship between the different molecules and regulation of Pgp ATPase activity. Five of the six compounds block VRP-stimulated ATPase activity (Figure 7), a behavior previously observed for other Pgp inhibitors including cyclosporin A (*2, 3*). Although not proven, one simple explanation for these findings is that the inhibition is competitive and that inhibitory compounds may compete for a binding site common to VRP. However, this is one but several possibilities, and more complex types of interactions could be involved, including allosteric inhibition or competition for a low-affinity and/or low-specificity site on the protein. In the case of the CP147673/674 pair, substitution of the tricyclic ring for a benzhydryl moiety restricts interaction at that site in an enantiomer-specific fashion. One possibility is that the tricyclic headgroup facilitates hydrophobic association of these compounds and that the difference in stereochemistry of the compounds due to chirality is no longer functionally relevant. The benzhydryl headgroup may be sufficiently different that the compounds bind to the protein in such a way that the chirality of the hydroxyl group becomes significant.

The analysis of the possible inhibitory properties of these compounds on VBL-stimulated ATPase activity suggested a more complex interaction. Two of the three basic structures (CP162398/399; CP147673/674) were observed to inhibit VBL-stimulated ATPase activity, but in an enantiomer-specific fashion, with the *S* enantiomer of each compound showing activity. Results from compounds CP172732/CP219994 suggest that altering the quinoline ring abrogates interaction at a VBL binding site. Whether this is due to direct competition for the VBL binding site or to a more indirect effect is presently not known. Despite the relatively small number of compounds tested in this study, the analysis of the compounds tested suggest intriguing functional differences in the well-established VBL and VRP stimulation of ATPase activity by Pgp. Indeed, the different inhibitory properties of the same compound for VBL- and for VRP-stimulated Pgp ATPase activity may suggest subtle but important differences in their binding site on Pgp, which may underlie subsequent stimulation of ATP hydrolysis by Pgp. For example, the two enantiomers CP162398/399 stimulate ATP hydrolysis by Pgp with very similar dose−response characteristics, and both inhibit VRP-stimulated Pgp ATPase. However, while the *R* enantiomer (CP162398) appears to

weakly stimulate VBL-mediated ATP hydrolysis, the *S* enantiomer (CP162399) produces a strong inhibition (4-fold reduction). On the other hand, both benzothiazoles (CP172732/CP219994) can stimulate equally ATP hydrolysis by Pgp, and both inhibit VRP-induced ATPase but are without effect on VBL-stimulated ATP hydrolysis by Pgp. Similarly, analysis of the benzhydryl-containing quinoline pair (CP1-47673/674) showed that they were both capable of stimulating ATPase activity as single agents but showed enantiomeric specificity for stimulating VBL- and VRP-mediated ATPase activity of Pgp.

Thus, these results suggest possible differences at the site of interaction of VBL and VRP on Pgp or possible functional differences in the way both molecules stimulate ATP hydrolysis by Pgp, these differences being revealed by our analysis of structurally related but distinct MDR modulators. We were somewhat surprised by these findings and possible interpretation. Indeed, direct transport studies have shown that VRP, like VBL, is transported by Pgp (*48*, *49*), and photoaffinity labeling studies show that the compounds compete with each other at a common or related binding site on Pgp (*50*, *51*). A rational discussion of the mode of action of the 1,4-disubstituted piperazine analogues tested here, including possible molecular interactions with known Pgp substrates and inhibitors such as VBL and VRP, awaits a further characterization of the binding sites of these molecules on Pgp.

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