0022-3565/99/2902-0854$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 290:854–862, 1999

Vol. 290, No. 2
Printed in U.S.A.

Selectivity of the Multidrug Resistance Modulator, LY335979, for P-Glycoprotein and Effect on Cytochrome P-450 Activities

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Accepted for publication February 22, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Overexpression of ATP-dependent drug efflux pumps, P-glycoprotein (Pgp) or multidrug resistance-associated protein (MRP), confers multidrug resistance to tumor cells. Modulators of multidrug resistance block the action of these pumps, thereby sensitizing cells to oncolytics. A potent Pgp modulator is LY335979, which fully sensitizes Pgp-expressing cells at 0.1 μM in cytotoxicity assays and for which Pgp has an affinity of 59 nM. The present study examines its effect on MRP1-mediated drug resistance and cytochrome P-450 (CYP) activity and its ability to serve as a Pgp substrate. Drug resistance was examined with HL60/ADR and MRP1-transfected HeLa-T5 cells. Drug cytotoxicity was unaffected by 1 μM LY335979; leukotriene C4 uptake into HeLa-T5 membrane vesicles was unaffected. Because the substrate specificity of Pgp and CYP3A overlap, the effect of LY335979 on the 1′-hydroxylation of midazolam by CYP3A in human liver microsomes was examined. The apparent K_i was 3.8 μM, ~60-fold higher than the affinity of Pgp for LY335979. The modulator's effect on Pgp was evaluated with Pgp-overexpressing CEM/vinblastine $(VLB)_{100}$ and parental CCRF-CEM cells. Both cell lines accumulated [^{3}H]LY335979 equally well and did not efflux [^{3}H]LY335979 during a 3-h incubation, indicating that it is not a substrate of Pgp. Equilibrium-binding studies with CEM/VLB_{100} plasma membranes and [^{3}H]LY335979 showed that Pgp had a K_d of 73 nM, which is in good agreement with the previously determined K_i value. Thus, LY335979 is an extremely potent Pgp, and not MRP1 or MRP2, modulator and has a significantly lower affinity for CYP3A than for Pgp.

Tumor cells become resistant to many structurally unrelated oncolytics by the overexpression of a membrane-associated P-glycoprotein (Pgp) or due to the overexpression of a related protein, the multidrug resistance-associated protein (MRP; Hill, 1996; Muller and Sarkadi, 1997). Both proteins are members of a superfamily of ATP-binding cassette (ABC) transport proteins that includes diverse members such as the cystic fibrosis transmembrane conductance regulator gene and the sulfonylurea receptor (Cole et al., 1992) as well as several homologs of MRP (Kool et al., 1997). Overexpression of Pgp and/or MRP1 or MRP2 results in an enhanced ability to export drugs from the cell, thereby conferring multidrug resistance (MDR; Keppler et al., 1999). In the case of Pgp, a number of noncytotoxic modulators have been developed that can be used in combination with oncolytics that prevent drugs from being effluxed by Pgp. These agents are able to sensitize multidrug-resistant cells to cancer agents to which they would otherwise be resistant (Ford and Hait, 1990).

One of the most potent Pgp modulators described to date is LY335979. This compound contains a cyclopropyldibenzosuberane moiety and sensitizes a number of Pgp-expressing resistant cell lines to oncolytics at 100 nM. LY335979 is an excellent modulator of Pgp-mediated MDR in the human lymphoblastic leukemia CEM/VLB_{100} cells and does not alter the drug sensitivity of parental CCRF-CEM cells to MDR oncolytics, such as doxorubicin, etoposide, paclitaxel, and VLB. Pgp has an affinity of 59 nM for LY335979 when measured by the displacement of VLB in equilibrium-binding studies. In addition, LY335979 enhances by 120 to 140% the survival of nude mice implanted with a murine Pgp-expressing leukemia cell line, P388/ADR. A distinguishing feature of this modulator is that it shows little to no alteration in the pharmacokinetics of doxorubicin, etoposide, or paclitaxel when administrated in combination with LY335979 in mice (Dantzig et al., 1996; Starling et al., 1997). Other Pgp modulators enhance plasma levels and decrease clearance of coadministered Pgp-pumped oncolytics (Gibaldi, 1992a,b; Lum et al., 1992; Sikic et al., 1997).

The cytochromes P-450 (CYP) are responsible for the majority of the oxidative metabolism of drugs and xenobiotics. Although approximately 15 forms of CYP participate in the metabolism of drugs, four forms, CYP3A4, CYP1A2, CYP2C9, and CYP2D6, together account for greater than 90% of the oxidative metabolism of drugs (Benet et al., 1996).

Received for publication November 10, 1998.

ABBREVIATIONS: Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; MDR, multidrug resistance; ABC, ATP-binding cassette; CYP, cytochrome P-450 enzyme; LTC_4, leukotriene C4; VLB, vinblastine.

Furthermore, CYP3A4 by itself participates in the metabolism of greater than 50% of drugs that are metabolized oxidatively. Interestingly, a large number of the substrates and modulators of Pgp also have been shown to be substrates or inhibitors of CYP3A4 (Wacher et al., 1995). Furthermore, Pgp-mediated transport of drugs and xenobiotics in the liver and intestine has been shown to influence CYP3A4 catalytic activity (Schuetz et al., 1996a,b). Thus, in the development of a Pgp modulator, knowledge of its effect on CYP3A4 catalytic activity is of great importance.

The present article examines whether LY335979 serves as a substrate of Pgp and examines the affinity of Pgp for the modulator when determined directly with radiolabeled ligand. The specificity of the modulator is examined for MRP1-mediated drug resistance as well as MRP2-mediated transport of leukotriene C4 (LTC_4). The effect of the modulator also is determined on four CYP enzymes, including CYP3A4, that are believed to be responsible for the metabolism of the majority of drugs in the liver.

Materials and Methods

Materials

LY335979 [(2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride] and LY329146 ([2-(4-bis-(methanesulfonyl)aminophenyl)-6-hydroxybenzo-[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone, free base) were obtained from Eli Lilly and Company (Indianapolis, IN). Radiolabeled [^{3}H]LY335979 was prepared as described previously by Czeskis (1997). [^{3}H]LTC_4 (110 Ci/mmol) and [^{3}H]VLB were purchased from DuPont/NEN (Boston, MA) and Moravek Biochemicals (Brea, CA), respectively. LTC_4, ATP, AMP, glutathione, creatine phosphate, creatine kinase, diclofenac, phenacetin, NADPH, *n*-butyric acid, and flunitrazepam were purchased from Sigma Chemical Co. (St. Louis, MO). $MgCl_2$ was purchased from EM Science (Cherry Hill, NJ). Growth medium and geneticin were purchased from Life Technologies (Grand Island, NY), and iron-supplemented bovine calf serum was purchased from Hyclone (Logan, UT). Midazolam and 1′-hydroxy midazolam were obtained from Hoffmann-La Roche (Nutley, NJ), and 4′-hydroxy diclofenac was obtained from Gentest Corp. (Woburn, MA). Bufuralol and 1′-hydroxy bufuralol were purchased from Ultrafine (Manchester, U.K.). Acetaminophen was obtained from Kodak (Rochester, NY). Meclofenamate was obtained from Cayman Chemical (Ann Arbor, MI).

Cell Lines

CCRF-CEM and CEM/VLB_{100} were provided by Dr. William T. Beck (Cancer Center, University of Illinois at Chicago, IL; Dantzig et al., 1996). The HL60 cell line panel was generously provided by Dr. Melvin Center (Kansas State University; Krishnamachary et al., 1994). The HeLa transfectants, the MRP1-transfected HeLa cells (HeLa-T5), and the vector control HeLa-C1 were obtained from Drs. Susan Cole and Roger Deeley (Queen's University, Kingston, Ontario, Canada; Grant et al., 1994; Almquist et al., 1995). These cells were grown as described previously. Human *MRP2*-transfected Madin-Darby canine kidney (MDCK-28) and the vector control-transfected MDCK (MDCK-K) were generously provided by Dr. Dietrich Cui (Deutsches Krebsforschungszentrum, Heidelberg, Germany; Keppler et al., 1999). Cells were grown to confluence for 2 days in minimal essential medium with Earle's salts containing L-glutamine, 10% fetal bovine serum, and 60 μg/ml geneticin. Before membrane vesicle preparation, cells were removed with trypsin and replated at confluence for 1 day in the same growth medium containing 10 mM *n*-butyric acid. Caco-2 cells were grown as described previously (Kuhfeld and Stratford, 1996).

Cellular Uptake and Efflux Studies

The methods for the accumulation and efflux of [^{3}H]LY335979 were as follows. The accumulation of LY335979 was assessed using the drug-sensitive parental cell line, CCRF-CEM, and the multidrug-resistant cell line, CEM/VLB_{100}. Cells were washed twice to remove the growth medium with Earle's balanced salt solution (Gibco, Grand Island, NY) buffered with 25 mM HEPES, pH 7.5 (300 mOsm/kg) (Trans-EBSS; flux buffer). Cells were resuspended to 2×10^7 cells/ml in Trans-EBSS. At time zero, cells were incubated at 37°C with either 1 μM [^{3}H]VLB (~0.03 μCi/nmol, final concentration) or 1 μM [^{3}H]LY335979 (~0.07 μCi/nmol, final concentration). Cells were kept suspended on an orbital shaker. At the indicated time period, cells were collected with a Brandel harvester onto a filter membrane (GF/C presoaked overnight with 0.3% polyethylenimine). The filters were washed with 1 ml of ice-cold Trans-EBSS five times and subsequently were removed for scintillation counting using Aquassure scintillation cocktail (Packard Instrument Co., Meriden, CT). Time points were measured in duplicate. Values were corrected for radioactivity bound to the filter without the addition of cells.

For efflux studies, cells were loaded for 2 h with the indicated drug and then diluted into flux buffer. The amount of radiolabeled drug that was retained by the cells was measured. Specifically, 2×10^6 cells were washed twice and incubated 0.5 h at 37°C in glucose-free Trans-EBSS, pH 7.5, containing 10 mM sodium azide, followed by incubation for 0.5 h in glucose-free Trans-EBSS containing 20 mM 2-deoxyglucose. Subsequently, cells were incubated at 37°C in 1 ml (96-well plate; precoated with 3% BSA) of either 1 μM [^{3}H]VLB or 1 μM [^{3}H]LY335979 for 2 h on an orbital shaker as described previously. To initiate efflux, cells were diluted 1:20 with Trans-EBSS, pH 7.5, which contains glucose. At the indicated time points, cells were collected onto a membrane filter as described above, and the amount of drug retained was measured by scintillation counting.

Permeability of Human Intestinal Caco-2 Epithelium

Caco-2 cells were grown on a porous membrane support and allowed to differentiate in culture to form a tight intestinal epithelium that expresses Pgp on the apical surface (Hunter et al., 1993; Kuhfeld and Stratford, 1996). LY335979 and VLB were examined for their ability to cross the epithelium. The nonradiolabeled compound was present in either the apical or basolateral compartment, and samples were removed from the opposing compartment over a 3-h time period. Samples were analyzed by HPLC for the presence of LY335979 or VLB. Specifically, 50-μl samples were injected onto a Zorbax SB-C8 column (15 cm × 4.6 mm; Rockland Technologies Inc., Palo Alto, CA) and eluted with an isocratic system (0.065% trifluoroacetic acid/35% acetonitrile) at a rate of 1.2 ml/min. The eluant was monitored at 210 nm; the retention times were 320 s for LY335979 and 225 s for VLB. The concentrations were calculated from a standard curve using the peak areas for each compound. An apparent permeability coefficient was calculated from the data (Kuhfeld and Stratford, 1996).

Cytotoxicity Assays

Cytotoxicity assays were performed using a panel of cell lines derived from the human promyelocytic leukemia cell line HL60/S by drug selection or human HeLa cells transfected with MRP1. The drug-sensitive parental line HL60/S and two multidrug-resistant lines, HL60/Vinc and HL60/ADR, were examined that overexpress either Pgp or MRP1, respectively. The HL60 cells were cultured with RPMI 1640 growth medium containing 25 mM HEPES (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and 50 μg/ml gentamycin. For the cytotoxicity assay, cells were washed once with culture medium and plated in 96-well culture dishes with 20,000 cells/well. The concentration of doxorubicin (Sigma) was varied from 0.001 to 10 μg/ml in the presence of the indicated final concentration of LY335979. Cells were grown for 48 h, and cell viability was determined with a Cell Titer 96 Aqueous Nonradioac-

tive Cell Proliferation Assay (Promega, Madison, WI). Alternatively, for the cytotoxicity assay with the HeLa transfectants, the MRP1-transfectant HeLa-T5, and the vector control HeLa-C1, cells were grown as described for the HL60 except the cells were permitted to attach for 24 h before drug treatment. The concentration of doxorubicin was varied from 0.02 to 10 μg/ml in the presence of the indicated final concentration of the test modulator. Cells were grown for 72 h, and cell viability was determined as described above (Dantzig et al., 1996). In both cases, the assay was performed in triplicate. The IC_{50} concentration was calculated as micrograms per milliliter.

Preparation of Membrane Vesicles

For the membrane vesicle preparations, a modification was used of previously published methods (Cornwell et al., 1986; Doige and Sharom, 1992). HeLa-T5 cells ($\sim 10^9$) were collected in ice-cold isotonic Tris buffer (250 mM sucrose, 0.2 mM $CaCl_2$, and 50 mM Tris-HCl, pH 7.5) with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM leupeptin, and 0.3 μM aprotinin) and disrupted by nitrogen cavitation after equilibration at 350 psi for 1 h in a bomb (Parr Instrument Company, Moline, IL). Membranes were isolated as described previously (Dantzig et al., 1996) and stored under argon in liquid nitrogen. This procedure was modified for the preparation of membrane vesicles from the human MRP2-transfected MDCK cell line and its vector control. Cells were lysed by washing and scrapping cells into a 1-mM sodium bicarbonate and subsequently diluted with the Tris buffer containing protease inhibitors as described above. The lysed cells were centrifuged at 1800*g* for 5 min and treated as described above to isolate the membrane vesicles.

Equilibrium Binding

Plasma membranes were prepared from CCRF-CEM and CEM/VLB_{100} cells as reported previously (Dantzig et al., 1996). Equilibrium binding for VLB was measured as reported (Dantzig et al., 1996). The time required for equilibrium binding for LY335979 was 60 min; nonspecific binding of LY335979 was measured in the presence of 50 μM LY335979. Binding was independent of the osmolarity of the incubation buffer.

LTC_4 Uptake by Membrane Vesicles

ATP-dependent transport of LTC_4 into membrane vesicles was measured by a modified rapid-filtration method that was adapted to a 96-well, microtiter dish format (Leier et al., 1994). The assay was conducted at 37°C in a total volume of 50 μl containing 3 to 5 μg of membrane vesicle protein, 50 nM [^{3}H]LTC_4, 10 mM $MgCl_2$, 1 mM glutathione, 4 mM ATP or AMP, 250 mM sucrose, and 50 mM Tris-HCl, pH 7.5, with an ATP-regenerating system consisting of 100 μg/ml creatine kinase and 10 mM creatine phosphate. Test compounds were dissolved in dimethyl sulfoxide (DMSO) with the final concentration of 5% DMSO present in both the test assay and in the control. MRP1-mediated uptake was measured for 1 min and stopped by washing three times with 200 μl ice-cold buffer followed by three more 1-ml washes using the Packard Filtermate 196 onto a unifilter-96 GF/B plate (Packard Instrument Co.). MRP2-mediated uptake was measured for 20 min at 37°C as described above, except 15 μg of membrane vesicles and 1% DMSO final concentration were used and uptake was measured for 20 min. The filter plates were dried overnight and sealed on the bottom with Packard backing tape before the addition of 40 μl of Microscint 20 (Packard Instrument Co.). Tritium was counted on a Top Count (Packard Instrument Co.). ATP-dependent uptake of [^{3}H]LTC_4 was calculated by subtraction of uptake measured in the presence of AMP. Each assay was measured in triplicate.

CYP Assays and Kinetic Analyses

Human livers designated HLB, HLH, HLM, HLO, and HLP were obtained from five individuals from the liver transplant unit at the Medical College of Wisconsin (Milwaukee) or Indiana University School of Medicine (Indianapolis) under protocols approved by the appropriate committees for the conduct of human research. Microsomes were prepared by differential centrifugation (van der Hoeven and Coon, 1974). A mixture of equal protein concentrations of microsomes from HLB, HLH, HLM, and HLP was prepared and used in the studies involving CYP2C9, CYP2D6, and CYP1A2. Microsomes from human liver sample HLO were used in the study involving CYP3A because previous studies demonstrated that this liver specimen contains high levels of CYP3A4 without detectable levels of CYP3A5 (Wrighton and Ring, 1994). The following assays were performed.

CYP3A. Microsomal incubations and HPLC analyses with the CYP3A substrate, midazolam, were performed as described previously (Wrighton and Ring, 1994). Incubations of midazolam (5, 10, 25, 50, or 100 μM) with human liver microsomes were performed with or without the addition of LY335979 (6.25, 12.5, 25, or 50 μM) as the inhibitor.

CYP2D6. Microsomal incubations and HPLC analyses with the CYP2D6 substrate bufuralol were carried out as described previously (Ring et al., 1996) with the following modification: 1 mM NADPH was used instead of a generating system. Incubations of bufuralol (5, 10, 25, 50, or 100 μM) with human liver microsomes were performed with or without the addition of LY335979 (10, 25, 50, or 75 μM) as the inhibitor.

CYP2C9. Diclofenac metabolism to 4′-OH diclofenac was used as a form-selective catalytic activity for human CYP2C9. Incubation mixtures of 200 μl contained human liver microsomes (0.05 mg) in 100 mM sodium phosphate (pH 7.4), 1 mM NADPH, and diclofenac (2.5, 5, 10, 25, or 50 μM) in the presence or absence of 10, 25, 50, or 75 μM LY335979 as an inhibitor. The reaction was stopped after 15 min with 200 μl of acetonitrile. Meclofenamate (internal standard) was added in a 10-μl volume. The denatured protein was removed by centrifugation, and the supernatant was subjected to HPLC analysis. Formation of 4′-OH diclofenac was measured by HPLC using a linear gradient from 80% mobile phase A (50 mM sodium phosphate, pH 7.4, containing 0.03% triethylamine) to 60% A. Mobile phase B consisted of acetonitrile. A volume of 50 μl of supernatant was injected onto a Betabasic C18 column (50 $\times$ 4.6 mm, 5 μm; Keystone Scientific, Bellefonte, PA) and monitored by UV detection at 282 nm. The flow rate was 1 ml/min, the total run time was 15 min, and chromatography was carried out at approximately 35°C.

CYP1A2. The CYP responsible for the biotransformation of phenacetin to acetaminophen (phenacetin *O*-de-ethylation) has been shown to be CYP1A2. Incubation mixtures of 200 μl contained human liver microsomes (0.1 mg) in 100 mM sodium phosphate, pH 7.4, 1 mM NADPH, and phenacetin, at a concentration near its K_m for CYP1A2 (12.5 μM) in the presence or absence of 5, 10, 25, or 50 μM LY335979 as an inhibitor. The reaction was stopped after 30 min with 200 μl of methanol. The denatured protein was removed by centrifugation, and the supernatant was subjected to HPLC analysis. The HPLC analysis of acetaminophen used UV detection at 254 nm. An Alltima phenyl column (150 $\times$ 4.6 mm, 5 μm; Alltech, Deerfield, IL) was used. The mobile phase consisted of 25 mM sodium phosphate buffer (pH 3.0)/methanol (95:5, v/v) and was delivered at a flow rate of 1.0 ml/min. Chromatography was carried out at approximately 35°C.

The apparent kinetic parameters of K_m, V_{max}, and K_i were determined by nonlinear regression analysis using NONLIN, version VO2-G-VAX (Statistical Consultants, Inc., Lexington, KY), as described by Ring et al. (1996).

Results

Accumulation and Efflux of LY335979. To determine whether LY335979 is a possible substrate for Pgp-mediated efflux, accumulation studies were conducted using radiola-

beled LY335979 or VLB in drug-sensitive CCRF-CEM and multidrug-resistant CEM/VLB_{100} cells. The data in Fig. 1 (lower) show that the uptake of [^{3}H]LY335979 into human leukemia cells is not affected by the presence of Pgp. By contrast, [^{3}H]VLB, a known substrate of Pgp, accumulated to a much lesser extent in the Pgp-expressing cell line (CEM/VLB_{100}) than in the parental cells (CCRF-CEM) during a 3-h time course (Fig. 1, top). These data suggest that LY335979 is not a substrate of Pgp. To verify this, both drug-sensitive and drug-resistant cells were loaded for 2 h with [^{3}H]LY335979 or [^{3}H]VLB in glucose-free buffer containing sodium azide and 2-deoxyglucose to deplete intracellular ATP. This permitted both cell types to accumulate equal concentrations of the labeled drug (data not shown). Cells then were diluted 20-fold into flux buffer containing glucose, and the amount of LY335979 or VLB retained intracellularly was measured over a 3-h time course. Figure 2 illustrates that both drug-sensitive and drug-resistant cells were unable to efflux LY335979, whereas multidrug-resistant CEM/VLB_{100} cells removed [^{3}H]VLB much more efficiently than drug-sensitive CCRF-CEM cells.

To confirm these results, the transport of 30 μM LY335979 was examined in a human intestinal cell culture model, Caco-2, that forms a differentiated tight epithelium when grown in culture. The intestinal Caco-2 epithelium expresses

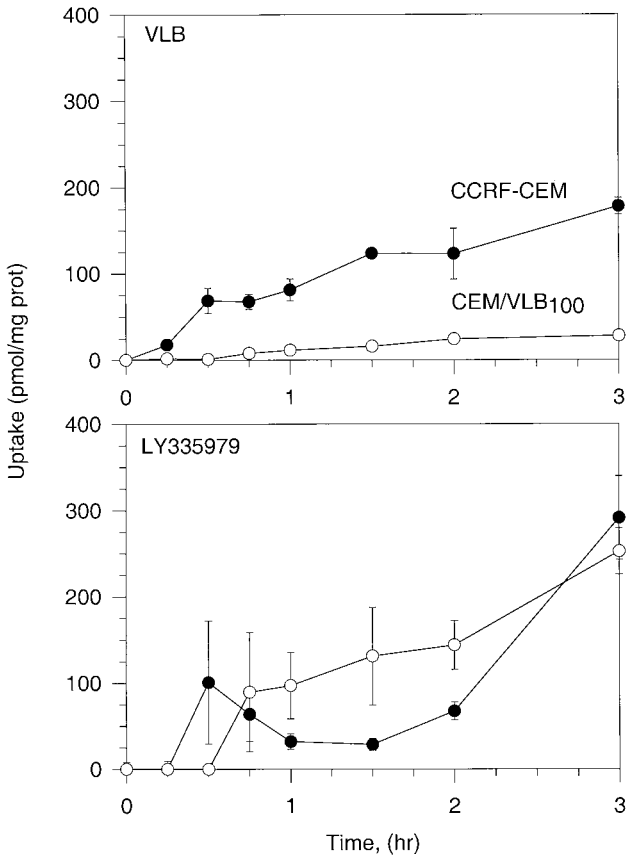

Fig. 1. Accumulation of VLB and LY335979 by drug-sensitive CCRF-CEM (●) and multidrug-resistant CEM/VLB_{100} (○) cells. Cells were incubated with either 1 μM [^{3}H]VLB (upper) or 1 μM [^{3}H]LY335979 (lower). Data are the mean ± S.D. of quadruplicate determinations. Differences in the accumulation of LY335979 in this experiment did not indicate that it was statistically significant (P = .44) in Student's t test. Curves are representative of three independent experiments.

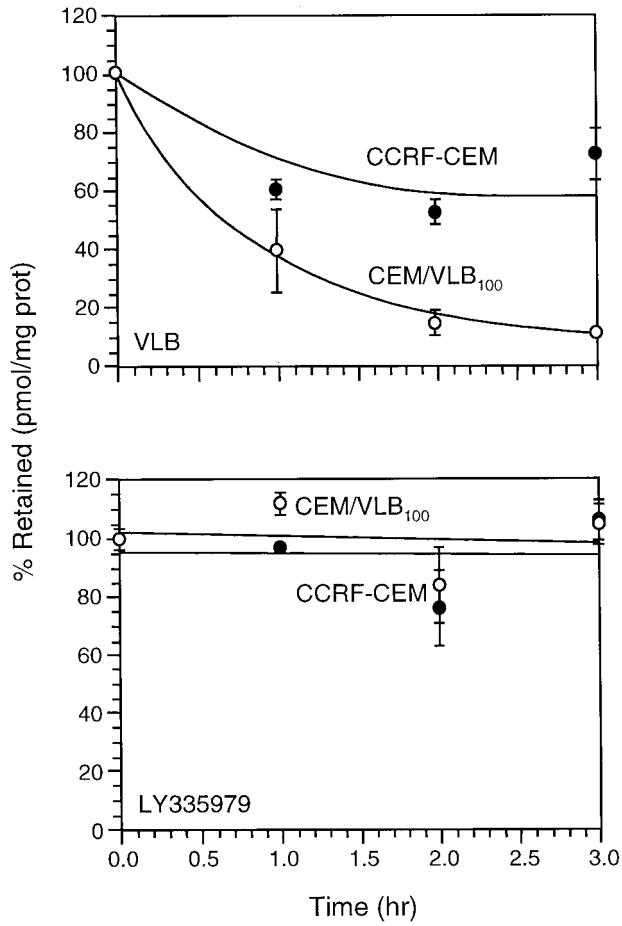

Fig. 2. Efflux of VLB or LY335979 from drug-sensitive CCRF-CEM (●) and multidrug-resistant CEM/VLB_{100} cells (○). Cells were incubated with either 1 μM [^{3}H]VLB (top) or 1 μM [^{3}H]LY335979 (lower) for 2 h in the presence of sodium azide and 2-deoxyglucose before being diluted 20-fold into flux buffer containing glucose. The amount of radiolabeled material retained by the cells was measured over a 3-h time course. Data are the average of two determinations. Curves are representative of two independent experiments.

Pgp on the apical membrane surface similar to the normal gastrointestinal epithelium and can be grown on a porous membrane support (Kuhfeld and Stratford, 1996). The Pgp substrate, VLB, has been shown to be transported asymmetrically across the epithelium from the basolateral to the apical side of the monolayer (Hunter et al., 1993). VLB and LY335979 were examined for their ability to cross the epithelium when presented individually in either the apical or basolateral compartment. Samples were removed over a 3-h time period from the opposing compartment. As shown in Fig. 3, VLB is transported vectorially across the monolayer with greater flux in the basolateral-to-apical direction than in the apical-to-basolateral direction. The permeability coefficients differed significantly in the two directions. The permeability coefficient was 21 $\times$ 10^{-5} cm/min in the apical-to-basolateral direction and 116 $\times$ 10^{-5} cm/min in the basolateral-to-apical direction, consistent with VLB being a substrate of Pgp and Pgp being expressed on the apical surface. By contrast, the flux of 30 μM LY335979 was equivalent in both directions as shown in Fig. 3 (lower). The permeability coefficients were determined to be 382 $\times$ 10^{-5} and 378 $\times$ 10^{-5} cm/min in the apical-to-basolateral direction and baso-

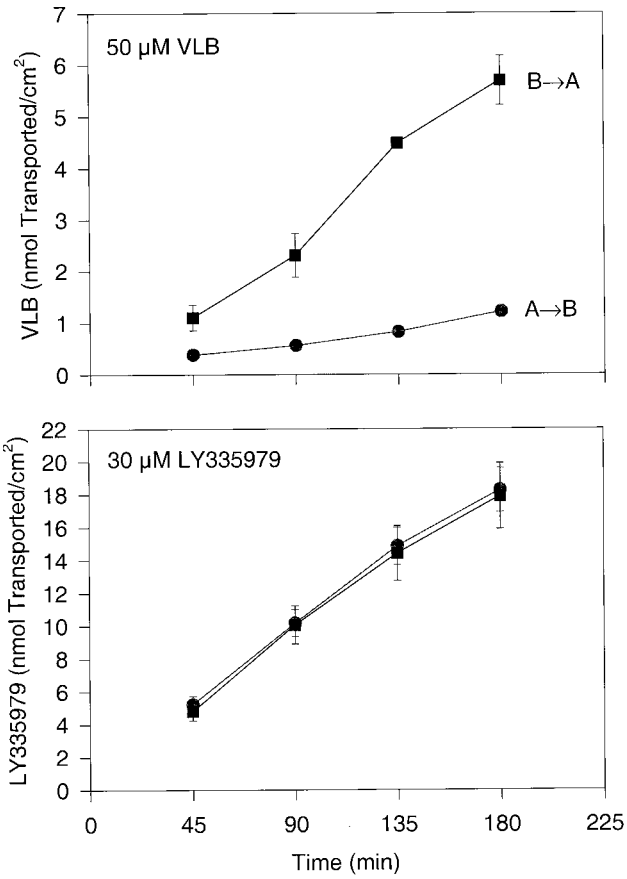

Fig. 3. Transport of VLB or LY335979 across the human intestinal Caco-2 epithelium. Appearance of the indicated compound into the opposite chamber was examined over a 180-min time course. Top, vectorial transport of 50 μM VLB to the opposite side of the monolayer. A $\rightarrow$ B represents apical-to-basolateral transport, and B $\rightarrow$ A represents the basolateral-to-apical transport. Bottom, transport of 30 μM LY335979 to the opposing compartment. Compounds were presented either on the basolateral side, and appearance was measured in the apical compartment (■), or on the apical side, and appearance was measured in the basolateral compartment (◆). Data are the mean ± S.E. of three determinations. The curves are representative of three independent experiments.

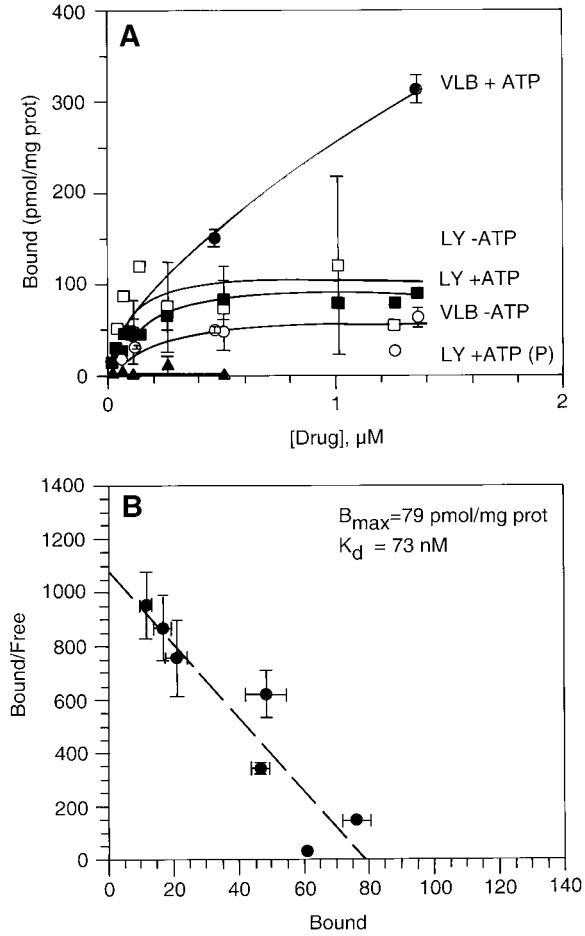

Fig. 4. Equilibrium binding to Pgp. A, the binding of [^{3}H]VLB and [^{3}H]LY335979 to CEM/VLB$_{100}$ membrane vesicles was measured over the indicated concentration range. Also shown is the binding of [^{3}H]LY335979 to the parental (P), drug-sensitive CCRF-CEM membrane vesicles measured in the presence of ATP. Data are the mean ± S.E. of nine data points obtained in three independent experiments, each measured in triplicate. B, Scatchard plot of binding of LY335979 to CEM/VLB$_{100}$ membrane vesicles measured in the presence of ATP. The calculated K_d is 73 nM, and B_{max} is 79 pmol/mg protein.

lateral-to-apical direction, respectively. Taken together, these studies indicate that LY335979 is not a substrate of Pgp-mediated efflux.

Binding to Pgp. Equilibrium-binding studies were conducted to compare the ability of Pgp to bind [^{3}H]LY335979 and [^{3}H]VLB using plasma membrane vesicles prepared from the Pgp-expressing, multidrug-resistant CEM/VLB$_{100}$ cells. Binding was measured in the absence or presence of ATP over a wide concentration range as shown in Fig. 4A. VLB binding was much higher in the presence of ATP than in the absence of ATP. By contrast, the binding of [^{3}H]LY335979 to CEM/VLB$_{100}$ membranes was quite similar when measured in the presence or absence of ATP, although it saturated with increasing drug concentrations. When binding was compared between membrane vesicles from multidrug-resistant CEM/VLB$_{100}$ and the drug-sensitive, parental CCRF-CEM cells, the binding of [^{3}H]LY335979 to parental membrane vesicles was negligible when compared with that of Pgp-expressing CEM/VLB$_{100}$ vesicles measured in the presence of ATP (Fig. 4A). This indicates that LY335979 binds to Pgp specifically as was observed previously for VLB binding (Dantzig et al., 1996). As shown in Fig. 4B, a Scatchard plot of the [^{3}H]LY335979-binding data obtained in the presence of ATP indicated the presence of a single binding site with a K_d of 73 nM and a B_{max} of 79 pmol/mg protein. The kinetic parameters for VLB binding were, respectively, K_d = 3 μM and B_{max} = 726 pmol/mg protein in the presence of ATP (Horio et al., 1988) and K_d = 0.31 μM and B_{max} = 85 pmol/mg protein in the absence of ATP (plots not shown). Interestingly, when binding of VLB is measured in the absence of ATP, the B_{max} was 85 pmol/mg protein, which was close to that of LY335979, which was 79 pmol/mg protein, measured in the presence of ATP.

Lack of Modulation of MRP. MDR also can result from the overexpression of MRP1, another member of the ABC transporter family (Cole et al., 1992). Overexpression of either Pgp or MRP1 confers resistance to doxorubicin and vincristine. To examine the selectivity of LY335979 for modulating MDR, a panel of HL60 cells was used. The vincristine-selected HL60/Vinc cells overexpress Pgp and not MRP1, whereas the doxorubicin-selected HL60/ADR cells

overexpress MRP1 and not Pgp (McGrath et al., 1989; Krishnamachary et al., 1994). Table 1 shows the effect of the modulator on the cytotoxicity of doxorubicin to these selected cell lines and the drug-sensitive HL60/S parental cell line. The modulator had no effect on the cytotoxicity of doxorubicin to the parental HL60/S cells and the MRP1-expressing HL60/ADR cells. The cytotoxicity of doxorubicin to the Pgp-expressing HL60/Vinc cells was enhanced by 28- to 62-fold in the presence of 0.01 to 1 μM LY335979, within the noncytotoxic concentration range of the modulator. The effect of LY335979 was also examined on the cytotoxicity of doxorubicin and vincristine to HeLa cells that were transfected with MRP1 (Table 2). The HeLa transfectants, HeLa-C1 and HeLa-T5, are, respectively, the drug-sensitive cells containing the vector only and the drug-resistant transfectant that expresses MRP1. HeLa-T5 cells exhibit a low level of drug resistance as shown in Table 2 for both doxorubicin and vincristine. The drug sensitivity of the cells was enhanced by the presence of 5 μM LY329146, a MRP1 modulator (Norman et al., 1997) and not by the presence of 1 μM LY335979, the Pgp modulator, in the growth medium. Moreover, membrane vesicles were prepared from MRP1-expressing HeLa-T5 cells, and the transport of a MRP1 substrate, LTC_4, was examined as shown in Fig. 5. The uptake of 50 nM [^{3}H]LTC_4 was inhibited by 5 μM MK571, a known inhibitor of the MRP1 transporter, but not by 5 μM LY335979. Furthermore, LTC_4 uptake was inhibited by the anti-MRP1 monoclonal antibody, QCRL-3, that binds to an intracellular epitope known to be critical for MRP1 transport function and was not inhibited by QCRL-1, which binds to another intracellular epitope that is not important for MRP1 transport function (Hipfner et al., 1992; Loe et al., 1996). These studies confirm that LY335979 is not a modulator of MRP1.

The canalicular-multispecific organic anion transporter has been shown recently to be a member of the ABC transporter superfamily and to have 49% identity to MRP1 and is also called MRP2 or cMRP (Keppler and Konig, 1997). Because of their strong homology and the important role of this transporter in organic anion efflux for drug conjugates in the liver, we wondered whether LY335979 might be an inhibitor of this transporter. Using MDCK cells that were transfected with human *mrp2*, membrane vesicles were prepared and the transport of LTC_4 was measured. As shown in Fig. 6, the rate of 50 nM [^{3}H]LTC_4 uptake was inhibited significantly by 10 μM MK571 but not by 10 μM LY335979 in the MDCK cells transfected with an empty vector or with *mrp2*. The difference between the uptake rates by membrane vesicles prepared from these two cell lines represents the uptake rate mediated by MRP2. The rate of MRP2-mediated uptake was not inhibited by the presence of 10 μM LY335979 (Fig. 6). Taken together, these data indicate that LY335979 is not a modulator of MRP1 or MRP2.

Effect on Human CYPs. A large number of Pgp substrates are known to interact with CYP3A. Therefore, the substrate specificities of Pgp and CYP3A appear to overlap (Wacher et al., 1995). Thus, LY335979 was examined for its ability to inhibit form-selective catalytic activities of not only CYP3A4 but also CYP2D6, CYP2C9, and CYP1A2. The type of inhibition of these enzymes by LY335979 was modeled using nonlinear regression analyses as indicated in *Materials and Methods*, yielding apparent K_m, V_{max}, and K_i values.

Midazolam metabolism to 1′-hydroxy midazolam is catalyzed by CYP3A4 (Wrighton and Ring, 1994). The formation of 1′-hydroxy midazolam followed simple Michaelis-Menten kinetics with an apparent K_m of 3.9 $\pm$ 0.7 μM and a V_{max} of 6177 $\pm$ 276 pmol product/min/mg protein. The best-fit model for inhibition of CYP3A4 by LY335979 was found to be competitive, yielding a K_i value of 3.8 $\pm$ 0.8 μM (Fig. 7 and Table 3).

The 1′-hydroxylation of bufuralol has been shown to be catalyzed by CYP2D6. The formation of 1′-hydroxy bufuralol was found to follow simple Michaelis-Menten kinetics, yielding an apparent K_m of 10.6 $\pm$ 1.2 μM and a V_{max} of 181 $\pm$ 8 pmol product/min/mg protein. The inhibition of CYP2D6 by LY335979 was found to model best as noncompetitive inhibition, yielding an apparent K_i of 25.3 $\pm$ 2.7 μM (Table 3).

The low-K_m human CYP responsible for the biotransformation of diclofenac to 4′-hydroxy diclofenac has been shown to be CYP2C9. The kinetics of formation of 4′-hydroxy diclofenac in the study with LY335979 yielded apparent K_m and V_{max} values of 2.4 $\pm$ 0.5 μM and 827 $\pm$ 48 pmol product/min/mg protein, respectively. The best-fit model describing the inhibition of CYP2C9 was found to be competitive, yielding a K_i of 12.3 $\pm$ 3.0 μM (Table 3).

Phenacetin de-ethylation to acetaminophen has been

TABLE 1

Selectivity of modulation of MDR

A panel of HL60 cells were used to examine the effect of LY335979 on cytotoxicity of doxorubicin. Cells were the HL60/S parental line, the HL60/Vinc, a Pgp-overexpressing cell line, and the HL60/ADR, MRP1-overexpressing cell line.

[LY335979]	Doxorubicin IC_{50} (fold-shift)		
	HL60/S	HL60/Vinc	HL60/ADR
μM		$\mu g/ml$	
0	0.04	3.65	3.67
0.01	0.04	0.13 (27.7)	4.04 (0.9)
0.05	0.03	0.08 (45.6)	3.42 (1.1)
1.00	0.03	0.06 (61.7)	3.00 (1.2)

TABLE 2

Lack of modulation of MRP1-mediated MDR in HeLa-T5 transfectants

Transfectants of HeLa used. HeLa-C1 was the drug-sensitive vector control and HeLa-T5 was the MRP1 transfectant.

Drug	IC_{50}			
	Doxorubicin		Vincristine	
	HeLa-C1	HeLa-T5	HeLa-C1	HeLa-T5
		$\mu g/ml$		
Control	0.38 $\pm$ 0.0	2.35 $\pm$ 0.22	0.006 $\pm$ 0.001	0.053 $\pm$ 0.005
LY329146 (5 μM)	0.27 $\pm$ 0.04	0.78 $\pm$ 0.11[a]	0.005 $\pm$ 0.002	0.016 $\pm$ 0.003[a]
LY335979 (1 μM)	0.52 $\pm$ 0.07	2.72 $\pm$ 0.08	0.006 $\pm$ 0.001	0.060 $\pm$ 0.014

[a] Significantly different from control by Student's t test ($P < .05$).

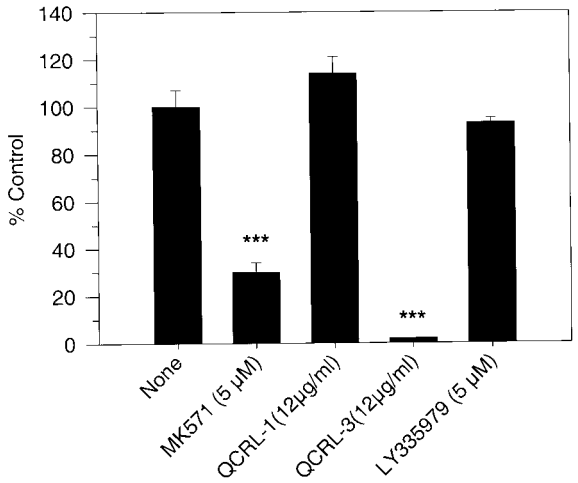

Fig. 5. Effect on 50 nM LTC_4 uptake into plasma membrane vesicles prepared from MRP1-transfected HeLa-T5 cells. Data are the mean ± S.E. of triplicate determinations. ***Significantly different by Student's t test ($P < .001$). Graph is representative of two independent experiments measured in triplicate.

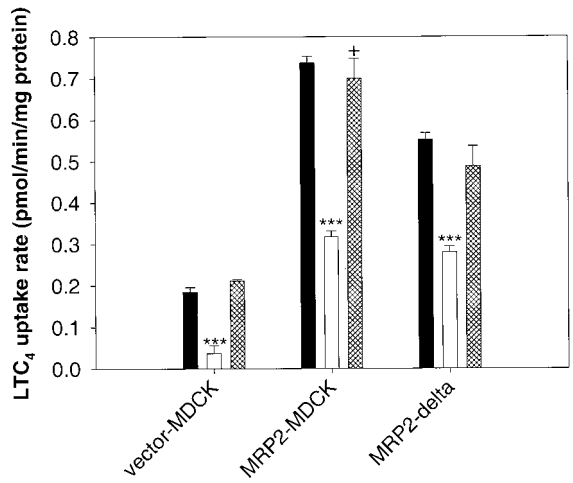

Fig. 6. Effect of LY335979 and MK571 on the rate of 50 nM LTC_4 uptake into plasma membrane vesicles prepared from MRP2-transfected MDCK and the vector-control MDCK cells. Uptake was measured for 20 min at 37°C in the absence of inhibitors (solid columns), in the presence of 10 μM MK571 (open columns), or in the presence of 10 μM LY335979 (shaded columns). MRP2-delta is the difference in the uptake rates measured with membrane vesicles prepared from MRP2-transfected and vector-control MDCK. Data are the mean ± S.E. of triplicate points. ***, data are significantly different by Student's t test ($P < .001$). +, data are not significantly different by Student's t test ($P > .5$). Graph is representative of three independent experiments.

shown to be catalyzed by CYP1A2. Acetaminophen formation from 12.5 μM phenacetin was only slightly (<16%) inhibited by concentrations of LY335979 as high as 50 μM (Table 3).

Discussion

The present study examines in more detail the in vitro properties of the potent Pgp modulator LY335979. Equilibrium-binding studies using radiolabeled LY335979 indicated that Pgp has a very high affinity for LY335979, with a K_d of 73 nM. This value is in excellent agreement with the previously reported K_i of 59 nM determined by the displacement of [^{3}H]VLB from Pgp in CEM/VLB_{100} membranes (Dantzig et al.,1996). Furthermore, comparison of the equilibrium binding of LY335979 in the absence or presence of ATP suggests that binding of the modulator is ATP-independent. The binding of VLB clearly is enhanced significantly in the presence of ATP as shown in Fig. 4A and as others have reported for vincristine (Naito et al., 1988); however, the presence of ATP has little effect on the binding of LY335979. In fact, the B_{max} of Pgp for LY335979 in the presence of ATP is quite similar to that of VLB measured in the absence of ATP, respectively: 79 pmol/mg protein and 85 pmol/mg protein. Because Pgp is likely to change conformations during its catalytic cycle (Stein, 1997; Shepard et al., 1998), these data suggest that LY335979 binds to a conformation of Pgp to which ATP is not bound. This may be the reason that LY335979 was found previously to have no effect on the ATPase activity associated with Pgp (Dantzig et al., 1996).

Studies with other modulators such as verapamil and cyclosporin A have suggested that these modulators may be transported by Pgp in certain cell lines (Stein, 1997). Studies were conducted to determine whether LY335979 is indeed a substrate of Pgp by comparing both the accumulation and efflux of a known substrate of Pgp, VLB, with LY335979 using drug-sensitive CCRF-CEM and multidrug-resistant CEM/VLB_{100} cells. Unlike the classic Pgp substrate, VLB, LY335979 accumulated similarly in both cell lines and was not effluxed significantly during a 3-h time course. Moreover, the transport of LY335979 across a monolayer of human

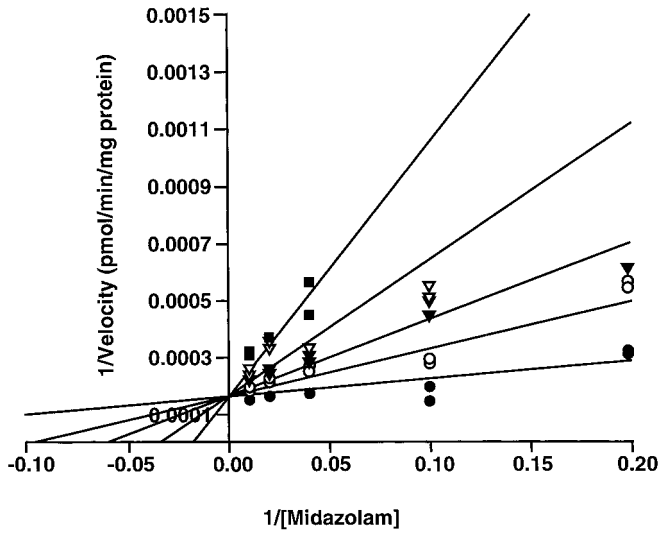

Fig. 7. Lineweaver-Burk representation of the inhibition of CYP3A-mediated 1′-hydroxy midazolam formation by LY335979. Each point represents a determination of velocity performed in duplicate at the indicated substrate concentration. The lines represent the best-fit model.

Caco-2 cells that forms a tight epithelium in culture and expresses Pgp on the apical membrane indicated that LY335979 is not transported vectorially across the epithelium even though VLB is transported vectorially. Collectively, these data indicate that LY335979 is not a substrate of Pgp. This may provide an explanation as to why LY335979 continues to modulate drug resistance for 3 h or longer in cytotoxicity assays after being washed from the cells, whereas verapamil rapidly lost its ability to modulate drug resistance after removal (Starling et al., 1997). A modulator that is not a substrate of Pgp may be expected to have a longer duration of action on tumor cells. Thus, LY335979 is a highly potent modulator, for which Pgp has an affinity of 59

TABLE 3
Effect of LY335979 on CYP3A, CYP2C9, CYP2D6, and CYP1A2 form-selective catalytic activities in vitro

CYP	Form-Selective Catalytic Activity	Type of Inhibition	Apparent K_i
			μM
CYP3A	Midazolam 1′-hydroxylation	Competitive	3.8 ± 0.8
CYP2D6	Bufuralol 1′-hydroxylation	Noncompetitive	25.3 ± 2.7
CYP2C9	Diclofenac 4′-hydroxylation	Competitive	12.3 ± 3.0
CYP1A2	Phenacetin *O*-de-ethylation		

[a] Form-selective biotransformation for CYP1A2, phenacetin *O*-de-ethylation, was inhibited only slightly (<20%) in the presence of up to 50 μM LY335979.

TABLE 4
Classes of natural-product oncolytics that are affected by Pgp, MRP1, and the CYP enzymes

	Vincas	Anthracyclines	Podophyllotoxins	Taxanes
Pgp	+	+	+	+
MRP1	+	+	+	
CYP3A	+	+	+	+
CYP2C				+

to 73 nM, that apparently binds to an ATP-independent conformation of Pgp and does not serve as a substrate of Pgp.

Several mechanisms have been demonstrated to be important in the removal and/or the metabolism and ultimate elimination of oncolytics in vivo as summarized in Table 4. Expression of both Pgp and MRP1 transport proteins in tissue or tumors results in the ATP-dependent efflux of oncolytics such as doxorubicin, vincristine, and etoposide. In addition, CYP isozymes are important in the oxidative metabolism of these drugs, which is necessary for their detoxification and ultimate elimination from the body. The taxanes are also transported by Pgp but not MRP1 and are metabolized by CYP isozymes. Because of the overlapping specificity of these transporters and CYPs, one might anticipate that a modulator of Pgp also could affect the activity of one or more of these proteins, potentially leading to drug-drug interactions. Lack of specificity of a modulator for Pgp could be expected to alter the metabolism by the CYPs and elimination by other transporters of one or more of these oncolytics.

Accordingly, the effect of LY335979 was examined on two other members of the superfamily of ABC transporters. MRP1-mediated resistance to either doxorubicin or vincristine was evaluated by examining the effect of LY335979 on the cytotoxicity of either MRP1-resistant cells that were selected continuously in culture with doxorubicin (HL60/ADR) or MRP1-transfected HeLa-T5 cells. LY335979 neither enhanced the cytotoxicity of doxorubicin in these cell lines nor inhibited the MRP1-mediated uptake of 5 nM LTC_4 into HeLa-T5 membrane vesicles. Moreover, the presence of 5 nM LY335979 was without effect on the uptake of LTC_4 by a close MRP1 homolog, MRP2, also known as canalicular-multispecific organic anion transporter or cMRP. Taken together, these data indicate that LY335979 is a potent, selective inhibitor of Pgp and is not a modulator of two other members of the ABC transporter superfamily, MRP1 and MRP2, at concentrations ~60-fold greater than those required to modulate Pgp.

When the effect of LY335979 was examined on four CYPs important in the metabolism of natural product oncolytics (Table 3), LY335979 was a competitive inhibitor of CYP3A with an apparent K_i of 3.8 μM when measured with the form-selective substrate midazolam in human liver microsomes (Fig. 7). The ability of LY335979 to inhibit the three other isozymes was even less with K_i values of 12 μM or greater. Thus, if the level of LY335979 reaches a concentration of 1 μM in vivo, the inhibition of CYP3A would be predicted to be 21% and inhibition of the other three isozymes would be 8% or less. Thus, LY335979 would be expected to have little effect on the pharmacokinetics of these oncolytics when LY335979 is dosed at levels that give concentrations equal to or below 1 μM. Dramatic effects on the plasma levels of the coadministered oncolytic have been observed with other Pgp modulators, such as verapamil, cyclosporin A, and PSC-833 (Gibaldi,1992a,b; Lum et al., 1992; Sikic et al., 1997). These Pgp modulators inhibit MRP1 and/or CYP isozyme(s) (Wandel et al., 1998). This lack of selectivity may be responsible, in part, for the observed drug-drug interactions that are observed in preclinical models and in clinical trials.

In conclusion, concentrations of LY335979 required to modulate the activity of Pgp would not be expected to alter the catalytic activity of the four major CYPs important in oncolytic metabolism or to modulate the transport activity of either MRP1 or MRP2. Therefore, LY335979 is a highly selective modulator of Pgp, and fewer pharmacokinetic drug-drug interactions are expected in vivo.

Acknowledgments

We thank Stacy Osborne, Li Liu, and Shannon Johnson for their excellent technical contributions to the Caco-2 studies.

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