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Reversal of P-Glycoprotein-mediated Multidrug Resistance by a Potent Cyclopropyldibenzosuberane Modulator, LY335979

Anne H. Dantzig,[1] Robert L. Shepard, Jin Cao, Kevin L. Law, William J. Ehlhardt, Todd M. Baughman, Thomas F. Bumol, and James J. Starling

Lilly Research Laboratories, Lilly Corporate Center, Eli Lilly and Company, Indianapolis, Indiana 46285-0424

ABSTRACT

Overexpression of P-glycoprotein (Pgp) by tumors results in multidrug resistance (MDR) to structurally unrelated oncolytics. MDR cells may be sensitized to these oncolytics when treated with a Pgp modulator. The present study evaluates LY335979 as a modulator both *in vitro* and *in vivo*. LY335979 (0.1 μM) fully restored sensitivity to vinblastine, doxorubicin (Dox), etoposide, and Taxol in CEM/VLB$_{100}$ cells. LY335979 modulated Dox cytotoxicity even when LY335979 (0.5 μM) was removed 24 h prior to the cytotoxicity assay. LY335979 blocked [^{3}H]azidopine photoaffinity labeling of the M_r ~170,000 Pgp in CEM/VLB$_{100}$ plasma membranes and competitively inhibited equilibrium binding of [^{3}H]vinblastine to Pgp (K_i of ~0.06 μM). Treatment of mice bearing P388/ADR murine leukemia cells with LY335979 in combination with Dox or etoposide gave a significant increase in life span with no apparent alteration of pharmacokinetics. LY335979 also enhanced the antitumor activity of Taxol in a MDR human non-small cell lung carcinoma nude mouse xenograft model. Thus, LY335979 is an extremely potent, efficacious modulator that apparently lacks pharmacokinetic interactions with coadministered anticancer drugs and is, therefore, an exciting new agent for clinical evaluation for reversal of Pgp-associated MDR.

INTRODUCTION

Tumors may be resistant to a number of oncolytics due to the expression of a plasma membrane-associated Pgp.[2] This protein is a transporter responsible for the energy-dependent efflux of a number of structurally unrelated natural product oncolytics that include vinblastine, doxorubicin, etoposide, and Taxol. Overexpression of this membrane protein allows MDR cells to efflux these drugs, thereby reducing the intracellular drug concentration and permitting the survival of tumor cells to drug concentrations that otherwise would be toxic. A number of non-cytotoxic agents (modulators) have been found that sensitize MDR cells to these oncolytics; several classes of modulators are known (1). First-generation modulators are drugs that were originally developed for other therapeutic indications. This generation includes calcium channel blockers such as verapamil; the immunosuppressive agents, cyclosporin A and FK506 (2); analogues of the antihypertensive drugs, reserpine and yohimbine; the neuroleptic, trifluoperazine; and antiestrogens, such as tamoxifen and toremifene. Second-generation modulators are agents developed that lack the original pharmacological activity of the first-generation compounds and usually possess a higher affinity for Pgp. These agents include the *R* isomer of verapamil, a nonimmunosuppressive analogue of cyclosporin D, SDZ PSC-833 (3, 4), a yohimbine analogue, TMBY (5), and others such as MS-073 (6), S-9788 (7), and GF120918 (8).

A novel potent modulator of Pgp, LY335979 (formerly called RS-33295-198), was described recently by Slate *et al.* (9) and Pfister *et al.* (10). This molecule (Fig. 1) contains a difluorocyclopropyl substitution in the dibenzosuberane moiety of MS-073 (6). LY335979 fully restored the doxorubicin sensitivity to several Pgp-expressing MDR cell lines when present at 12 to 100 nM in growth medium and also prevented the accumulation of doxorubicin intracellularly (9). Furthermore, LY335979 enhanced the survival time of mice inoculated with P388/ADR cells by 120–140%. The present study was undertaken to more completely characterize the ability of LY335979 to sensitize Pgp-expressing cells to oncolytics *in vitro*, to determine its interaction with Pgp, and to further evaluate its efficacy in the treatment of mice bearing MDR tumors. Experiments were also performed to evaluate the possible pharmacokinetic interactions between LY335979 and antitumor compounds. The results indicate that LY335979 is an extremely potent, efficacious modulator that apparently lacks pharmacokinetic interactions with coadministered anticancer drugs and is, therefore, an exciting new agent for clinical evaluation.

MATERIALS AND METHODS

Materials. [^{3}H]Vinblastine sulfate (9 Ci/mmol), [^{3}H]Taxol (19 Ci/mmol), and [^{3}H]etoposide (0.5 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA), and [^{3}H]azidopine (49 Ci/mmol) was purchased from Amersham International (Buckinghamshire, England). [^{3}H]Daunomycin (2.13 Ci/mmol) and [^{125}I]-labeled goat antimouse secondary antibody (2–10 μCi/μg) was purchased from DuPont NEN (Boston, MA). Vinblastine sulfate, etoposide, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO), and cyclosporin A was purchased from Sandoz Pharmaceuticals Corp. (East Hanover, NJ). Taxol was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA) and doxorubicin·HCl from either Sigma Chemical Co. or Meiji Seika Kaisha (Tokyo, Japan). Growth medium and cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY); FBS was purchased from Hyclone (Logan, UT). Biomedical Research and Development Laboratories (Gaithersburg, MD) was the supplier of the Brandel filters used for binding assay and Pierce Company (Rockford, IL) was the supplier for protein assay reagents. BDF$_1$ mice were purchased from Taconic (German Town, NY), and female nude mice were supplied by Charles River Breeding Laboratories (Boston, MA).

Cell Culture. Both the drug-sensitive parental cell line, CCRF-CEM, and the MDR cell line, CEM/VLB$_{100}$ (selected for resistance to 100 ng/ml VLB), were obtained from Dr. W. T. Beck (St. Judes Children's Research Hospital, Memphis, TN). Cells were maintained in MEM for suspension cultures containing Earle's salts, 2 mM L-glutamine, and 10% FBS (11). For the plasma membrane preparation, cells were grown in spinner flasks in medium supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin, and 141 μg/ml bacitracin. P388, P388/ADR, MCF-7, and MCF-7/ADR (DCT Tumor Repository, Frederick, MD) were grown in RPMI 1640 with L-glutamine, 10% FBS, and 50 μg/ml gentamicin. The human ovarian carcinoma cell lines, 2780 and 2780AD, were provided by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA) and were grown in RPMI 1640 containing 10% FBS, 2 mM glutamine, 10 μg/ml insulin, and 50 μg/ml gentamicin. UCLA-P3 human non-small cell lung carcinoma cells (12) were grown in RPMI 1640 containing 10% FBS and 50 μg/ml gentamicin. The cells were selected *in vitro* for resistance to 3 ng/ml desacetylvinblastine hemisuccinate over a 10-month time period. Pgp expression was determined by flow cytometry using the HYB-241 monoclonal antibody (13). The resistant cell line, UCLA-P3.003VLB, exhibited an approximate 8-fold increase in Pgp expression compared to the wild-type UCLA-P3 tumor cells.

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[1] To whom requests for reprints should be addressed. Phone: (317) 276-5083; Fax: (317) 276-9086.

[2] The abbreviations used are: Pgp, P-glycoprotein; LY335979, (2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride; MDR, multidrug resistant; FBS, fetal bovine serum; AUC, area under the curve; TMBY, trimethoxybenzoylyohimbine.

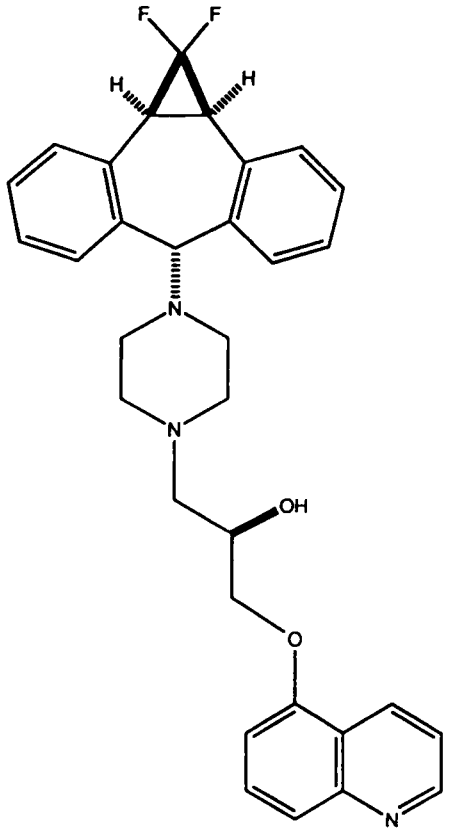

Fig. 1. Structure of the P-glycoprotein modulator, LY335979.

Cytotoxicity Assays. Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction method (14). Cells were harvested during logarithmic growth phase, and seeded in 96-well plates (Costar) at 1–7.5 × 10^4 cells/well. The cells were then cultured for 72 h in the presence of oncolytics with or without modulators. MCF-7 and MCF-7/ADR cells were incubated 24 h before the addition of the drug with and without the modulator. Modulators were prepared as 2 mM DMSO stocks and added to wells to give final concentrations ranging from 0.05 to 5 μM. After 72 h, 20 μl of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml in Dulbecco's PBS) was added to each well and incubated for 4 h in a 37°C incubator containing 5% CO_2. Cells were pelleted in a Sorvall RT6000B centrifuge, 70 μl of medium was carefully removed from each well, and 100 μl of 2-propanol/0.04 N HCl was added. Cells were resuspended 5–10 times with a Multipipettor or until no particulate matter was visible. Plates were immediately read on a Titertek Multiskan MCC/340 microplate reader Flow Laboratories (McLean, VA) with a test wavelength of 570 nm and a reference wavelength of 630 nm. Controls were measured in quadruplicate and modulators were measured in duplicate. Cytotoxicity analyses were also performed using the CellTiter 96 AQueous assay kit (Promega Corp., Madison, WI) according to the manufacturer's instructions.

Plasma Membrane Preparation. Plasma membranes were prepared by nitrogen cavitation and differential centrifugation. A total of 1–3.5 × 10^9 cells in logarithmic growth phase were centrifuged and washed as reported by Lever (15). The pellet was resuspended at ~3 × 10^7 cells/ml in 0.2 mM $CaCl_2$, 0.25 M sucrose, 0.02 mM phenylmethylsulfonyl fluoride, and 0.01 M Tris-HCl (pH 7.4). Cells were disrupted by nitrogen cavitation (Parr Instrument Co., Moline, IL) at 175 p.s.i. After removal of nuclei and unbroken cells by centrifugation, 1 mM EDTA was added to the supernatant and centrifuged at 9000 × *g* for 20 min to remove mitochondria. The resulting supernatant was layered onto a 35% sucrose gradient and centrifuged at 16,000 × *g* for 1 h as described (15). Membranes collected at the interface were subsequently pelleted at 100,000 × *g* for 1 h, resuspended in 0.20 M sucrose and 0.05 M Tris-HCl (pH 7.4), passed through a 25-gauge needle, and stored up to 2 months at −70°C. Protein was determined with bicinchoninic acid and BSA as the standard (16). The orientation of the membrane vesicles was estimated to be 95% inside-out, as determined by the activity of Na^+,K^+-ATPase measured in sealed and unsealed lyophilized vesicles.

Photoaffinity Labeling. Plasma membranes (12.5 μg protein) were photolabeled with 0.45 μM [^{3}H]azidopine (1.81 TBq/mmol) in the absence or presence of the indicated modulator in a U-shaped 96-multiwell dish (8). After incubation in the dark for 25 min at 25°C, the dish was placed on ice and irradiated for 2-min with a hand-held UV lamp (254 nm; UVP, Inc.) on top of the dish. Proteins were solubilized in electrophoresis sample buffer containing DTT (6 mg/ml) and heated to 37°C for 5 min prior to separation by a 8% SDS-PAGE (17). Subsequently, the gel was soaked in Amplify (Amersham) for 15 min, dried, and exposed to DuPont Cronex-4 film at −70°C. The amount of Pgp labeled in each band was quantitated from the fluorograms with a Truvel Bioimage scanner.

Equilibrium Binding. A rapid filtration method was used to determine equilibrium binding to plasma membranes. Routinely, CEM/VLB_{100} plasma membranes (~20 μg protein) were incubated in 200 μl total volume of 0.20 M sucrose, 3 mM ATP, 1 mM $MgCl_2$, and 0.05 M Tris-HCl (pH 7.4) (Buffer A) containing 0.1% BSA and either 40 nM [^{3}H]vinblastine, 40 nM [^{3}H]Taxol, or 2 μM [^{3}H]etoposide. The assay mixture was incubated at 25°C in 1-ml polystyrene plates (Beckman 96-well; soaked overnight with 3% BSA). After 150 min, plasma membranes were aspirated onto membrane filters (Brandel GF/C, soaked overnight in 10% FBS, 0.02 M sucrose, and 0.01 M Tris-HCl, pH 7.4) with a 48-channel cell harvester (Brandel, Inc., Gaithersburg, MD) and rapidly washed five times with 1 ml ice-cold Buffer A (18, 19). The wash buffer contained the same concentration of the indicated nucleotide as in the incubation condition. IC_{50}s were determined. For VLB, the total binding was corrected for nonspecific binding measured in the presence of 400 μM VLB. For both Taxol and etoposide, IC_{50}s were estimated from the maximum amount of radiolabeled oncolytic that could be displaced by the modulator. Unless noted otherwise, values are the mean ± SE of triplicate determinations.

ATPase Assay. P-Glycoprotein ATPase activity was measured by the liberation of inorganic phosphate from ATP using the Fiske and Subbarow method (20). The assay was measured in a 96-well plate for 90 min at 37°C. Membranes (8–10 μg protein) were incubated in a total volume of 100 μl of buffer A containing 5 mM sodium azide, 1 mM ouabain, 1 mM EGTA, 3 mM ATP, an ATP regenerating system composed of 5 mM phosphoenolpyruvate, and 3.6 units/ml pyruvate kinase in the presence and absence of 1 mM sodium vanadate. Pgp-ATPase activity was defined as the vanadate-sensitive portion of the total ATPase activity. Plates were read 3 min after the addition of the detection solution. The absorbance was measured at 690 nm by a microtiter dish reader (Titertek Multiskan MCC/340). A phosphate standard curve was used to calculate the μmol of phosphate formed. Samples were measured in triplicate, and values are the mean ± SE.

Accumulation and Efflux of Daunomycin. Uptake and efflux studies were performed essentially by the procedures of Hyafil *et al.* (8). Briefly, for the uptake studies 40,000 2780AD tumor cells were incubated 24 h in a 96-well microtiter plate at 37°C, and the medium was removed and replaced by 50 μl of cell culture medium containing 0.4 μM [^{3}H]daunomycin and various concentrations of modulators. After an additional 2.5-h incubation at 37°C, the medium was aspirated, and the cells was lysed overnight at 4°C in 0.1 M NaOH. Efflux of [^{3}H]daunomycin was performed as described by Hyafil *et al.* (8) using 2780AD tumor cells and preloading the cells with 0.3 μM [^{3}H]daunomycin.

***In Vivo* Evaluation.** P388 or P388/ADR cells were maintained in RPMI 1640 containing 10% FBS and 50 μg/ml gentamicin and were passed twice weekly. Cells were washed with serum-free medium three times, and 1 × 10^6 cells were implanted by i.p. injection into female BDF_1 mice weighing 20–25 g. All drug treatments started on the same day of tumor implantation. In the i.p. injection model, doxorubicin (stock concentration was 1 mg/ml in PBS) and LY335979 (stock solution was 2.5 to 5 mg/ml in 5% mannitol) were freshly prepared and mixed together immediately prior to administration. In the i.v./i.p. injection model, mice received 20 mg/kg of LY335979 by i.v. administration, followed by an i.p. injection of the indicated antitumor drug 30 min later, and continued to be treated once daily in this fashion for 5 days. The survival of mice in each group was examined daily. The results were represented as the curve of number of survival mice *versus* days after tumor implantation. Data were analyzed by the Kaplan-Meier method, Wilcoxon signed-rank test, using the JMP Statistics Program (SAS Institute, Cary, NC).

Table 1 *Modulation of cytotoxicity of CEM/VLB$_{100}$ and CCRF-CEM cells*[a]

Modulator	μM	Vinblastine		Doxorubicin		Etoposide		Taxol	
		IC_{50}	fold shift	IC_{50}	fold shift	IC_{50}	fold shift	IC_{50}	fold shift
CEM/VLB100[b]									
None		114.8		328.8		4376.8		2682.4	
Verapamil	5	2.4	48	71.7	5	3076.2	1.4	116.3	23
TMBY	5	0.2	574	46.1	7	600.1	7	13.0	206
LY335979	2	0.3	383	29.2	11	220.8	20	1.6	1647
	0.5	0.3	383	24.1	14	197.7	22	1.8	1490
	0.1	0.3	383	24.7	13	229.7	19	2.3	1166
	0.05	0.8	144	27.5	12	553.6	8	5.6	479
CCRF-CEM									
None[c]		0.49		24.7		516.8		5.6	
TMBY[d]	2	0.45	<1	29.7	<1	ND		15.1	<1
LY335979[d]	2	1.4	<1	27.4	<1	ND		16.6	<1

[a] Values for IC_{50}s were determined as ng/ml.
[b] Values are the means determined in duplicate in two independent experiments.
[c] Values are the means determined in duplicate in three to eight independent experiments.
[d] Values are the average of duplicates from a single experiment; ND, not determined.

In vivo antitumor activity was also evaluated using a human tumor xenograft model. UCLA-P3.003VLB MDR tumor cells were grown in tissue culture and removed from the substratum with trypsin-EDTA. The cells were washed three times in PBS, and 1×10^7 tumor cells were implanted s.c. into the rear flanks of female nude mice (eight mice/group). Five days after tumor implantation, LY335979 or vehicle control was given i.p., followed 1 h later by an i.v. injection of vehicle control or Taxol (formulated in 5% ethanol, 5% Cremophor EL, and 0.8% saline). Tumor sizes were determined by caliper measurements, and tumor mass in milligrams was estimated from the formula $l \times w^2/2$ where l is the length and w is the width of the tumor mass in millimeters. Student's t test was used to assess for significance between treatment groups.

Pharmacokinetic Studies. Female BDF_1 mice (20–25 g) were given i.p. doses of 5 mg/kg doxorubicin or 30 mg/kg etoposide alone or in combination with the indicated MDR modulator (100 mg/kg cyclosporin A, 40 mg/kg verapamil, or 30 mg/kg LY335979). Dose solutions were combined in the same vial and given as one bolus i.p. dose as described above. Blood samples were taken, in heparinized syringes, by cardiac puncture at time points 0.5, 1, 2, 4, 6, and 8 h (three mice/group), and centrifuged to separate plasma, which was stored at −70°C until analysis. Plasma samples were assayed for levels of the indicated oncolytic. In a separate experiment, mice were given i.p. doses of 4 mg/kg doxorubicin, alone or in combination with 20 mg/kg LY335979 i.v. Plasma samples were assayed for levels of doxorubicin at time points 0.25, 1, 2, 6, 10, 14, and 18 h (three mice/time point) after five daily doses. Doxorubicin was measured with slight modification of a reverse-phase isocratic high-performance liquid chromatographic assay described previously (21). A 75-μl sample was injected directly into a 3.9 × 150-mm Waters Novapak phenyl column using fluorescence detection (excitation, 475 nm; total emission with wavelength cutoff was set at 530 nm). With a flow rate of 1.5 ml/min, the retention time was 4.2 min for doxorubicin and 9.7 min for daunomycin. The detection limit for doxorubicin was 20 ng/ml. Etoposide was measured using reverse-phase isocratic liquid chromatography by the method of Harvey *et al.* (22). The detection limit for etoposide was 50 ng/ml. AUC throughout the time interval was calculated from the plasma levels for each experiment.

RESULTS

Modulation of Drug Resistance. The effect of LY335979 on the cytotoxicity of four clinically used oncolytics, vinblastine, doxorubicin, etoposide, and Taxol, was examined. Five cell lines (CEM/VLB$_{100}$, MCF-7/ADR, 2780AD, P388/ADR, and UCLA-P3.003VLB) that are drug resistant due to an overexpression of Pgp were used for this purpose. The effectiveness of the modulator was calculated as the "fold shift," which is the ratio of the IC_{50} measured in the presence and the absence of the modulator (see "Materials and Methods") and was compared to two other Pgp modulators, verapamil and TMBY (5). As shown in Table 1, 5 μM verapamil enhanced the cytotoxicity of vinblastine, doxorubicin, and Taxol from 5- to 23-fold, whereas 5 μM TMBY enhanced the antitumor activity for all oncolytics by 7- to 506-fold. By contrast, LY335979 was an effective modulator, showing maximal activity in restoring the sensitivity of resistant cells to the various anticancer drugs at concentrations from 2 to 0.1 μM. The modulator activity of LY335979 was diminished roughly 50% at 0.05 μM. Moreover, the presence of 2 μM TMBY or LY335979 had little effect on the cytotoxicity of these oncolytics to

Table 2 *Modulation of cytotoxicity in several Pgp-expressing MDR cell lines and their parental drug-sensitive lines*[a]

Drug	IC_{50}[b] (fold shift)				
	P388/ADR			P388	
LY335979[c]	0	0.1 μM	0.5 μM	0	0.5 μM
Vinblastine	0.205	0.003 (68)	0.002 (103)	0.001	0.0004 (2.5)
Doxorubicin	4.099	0.088 (47)	0.063 (65)	0.072	0.037 (1.9)
Taxol	8.890	0.046 (193)	0.013 (684)	0.022	0.004 (5.5)
	MCF-7/ADR			MCF-7	
Vinblastine	2.15	0.0004 (5375)	0.0007 (3071)	0.001	0.0009 (1.1)
Doxorubicin	8.66	0.489 (18)	0.628 (14)	0.751	0.336 (2.2)
Taxol	15.28	0.017 (899)	0.008 (1910)	0.0059	0.0045 (1.3)
	2780AD			2780	
LY335979[c]	0		0.5 μM	0	5.0 μM
Doxorubicin	>10		0.24 (>42)	0.09	0.05 (1.8)
	UCLA-P3.003VLB			UCLA-P3	
Taxol	0.18		0.006 (30)	0.004	0.002 (2.0)

[a] MDR cell lines are listed in the left columns, and their respective parental cell lines are on the right.
[b] IC_{50}s are reported in μg/ml.
[c] Concentration of LY335939 present in the assay with the indicated oncolytic.

the drug-sensitive parental cell line, CCRF-CEM cells (Table 1). In a control experiment using CEM/VLB$_{100}$ cells, the presence of 0.1 or 0.5 μM LY335979 had little to no effect on the cytotoxicity of four other oncolytics that are not associated with Pgp-mediated multidrug resistance and to which these cells are not cross-resistant. There was no effect on either bleomycin or 5-fluorouracil cytotoxicity and only a 4- and 2-fold shift was observed, respectively, for mitomycin C and cisplatin. As illustrated in Table 2, LY335979 was evaluated for its ability to modulate cytotoxicity of the oncolytics (vinblastine, doxorubicin, or etoposide) to the MDR cell lines P388/ADR, MCF7/ADR, 2780AD, or UCLA-P3.003VLB. At 0.1 and 0.5 μM, the compound gave nearly complete reversal of resistance for these oncolytics. LY335979 displayed minimal modulating activity against the drug-sensitive parental cell lines P388, MCF-7, 2780, or UCLA-P3 cell lines when administered at either 0.5 or 5 μM with reversal factors of 1.3–5.9. Taken together, LY335979 was about 100 times more potent than verapamil and TMBY and is an effective modulator in both human and mouse Pgp-expressing cell lines.

Cytotoxicity of LY335979 Alone. To confirm that the enhanced cytotoxicity with the LY335979 seen in Tables 1 and 2 was due to modulation and not due to cytotoxicity of the agent itself, the IC$_{50}$ concentration was determined for both the parental and resistant cell lines used above (Table 3). Although the IC$_{50}$ concentration varied between cell lines, the value between the resistant and parent lines were similar if not identical. These data show that the concentration needed to reverse drug resistance *in vitro* is 100-fold lower than the cytotoxic concentration of LY335979.

Duration of Drug Effect. To evaluate the duration of action of LY335979 as a modulator, 2780AD cells were incubated with LY335979, washed to remove the modulator, and incubated for various times in the absence of the reversal agent prior to the addition of doxorubicin. Verapamil was used as a comparator at the highest non-cytotoxic concentration that can be used with these cells. Thus, cells were incubated with either 10 μM verapamil, 0.5 or 0.05 μM LY335979 for 24 h, washed three times, incubated in growth medium for 0 to 24 h, and exposed to varying doxorubicin concentrations for 24 h. Afterwards, cells were washed to remove doxorubicin and incubated for an additional 3 days prior to the determination of cell viability. Table 4 summarizes the results. The modulating activity of verapamil disappeared immediately after its removal from the medium. The reversal effect of 0.5 μM LY335979, on the other hand, persisted throughout the 24-h time period (Table 4). By contrast, when cells were incubated with 0.05 μM LY335979, a significant reduction in modulating activity was seen after washing. Under these conditions, equivalent or better activity to verapamil (without washing) was observed for up to 3 h, and modulator activity, although reduced, was observed for up to 12 h. It is especially noteworthy that, in contrast to verapamil, the concentrations of LY335979 used in this assay (0.5 and 0.05 μM) are well below the cytotoxic IC$_{50}$ of LY335979 against the 2780AD cell line (16 μM; Table 3).

Table 3 *Cytotoxicity of LY335979 alone to drug-sensitive and MDR cell lines*

Cells were grown as described in "Materials and Methods" in the presence of increasing concentrations of LY335979. The IC$_{50}$ was determined for both the drug-sensitive and drug-resistant cell lines.

Cell line	IC$_{50}$ (μM)
CCRF-CEM	6
CEM/VLB$_{100}$	7
P388	15
P388/ADR	8
MCF7	7
MCF7/ADR	15
2780	11
2780AD	16
UCLA-P3	>5
UCLA-P3.003VLB	>5

Table 4 *Reversibility of effect of verapamil and LY335979 on doxorubicin cytotoxicity to 2780AD cells*[a]

Treatment schedule	IC$_{50}$ (μg/ml)	
Control[b]	>10	
Verapamil	10 μM	
No wash, 0 h	2.4	
Wash, 0 h	>10	
LY335979	0.5 μM	0.05 μM
No wash, 0 h	0.18	0.26
Wash, 0 h	0.25	0.88
Wash, 1 h	0.28	2.2
Wash, 3 h	0.30	2.4
Wash, 5 h	0.36	4.9
Wash, 8 h	0.35	6.5
Wash, 12 h	0.49	6.2
Wash, 24 h	0.48	>10

[a] 2780AD cells (10^4) per well were grown 24 h in growth medium without drug. The indicated modulator was added at the stated concentrations (verapamil, 10 μM; LY335979, 0.5 or 0.05 μM) and incubated 24 h prior to being washed 0 or 3 times with growth medium. Cells were then incubated for 0, 1, 3, 5, 8, 12, or 24 h in the absence of the modulator or doxorubicin. Subsequently, varying concentrations of doxorubicin were added to the cells and incubated for 24 h. After this treatment, cells were washed and incubated in growth medium without drug or modulator for an additional 72 h prior to cytotoxicity analysis.

[b] In the control, cells were incubated as above, except no modulator was added during the 24-h incubation before doxorubicin was added.

Effect on Daunomycin Accumulation and Efflux. Transport assays were conducted to analyze the effect of the modulator on both the accumulation and efflux of [^{3}H]daunomycin. As illustrated in Fig. 2*A*, LY335979 significantly enhanced the 2.5-h accumulation of 0.4 μM [^{3}H]daunomycin into 2780AD MDR ovarian tumor cells at modulator concentrations down to 0.1 μM. Verapamil, on the other hand, increased [^{3}H]daunomycin accumulation only when present at 100 μM, the highest level tested. Fig. 2*B* shows that 0.5 μM LY335979 effectively inhibited the efflux of [^{3}H]daunomycin from 2780AD tumor cells when the modulator was present in the uptake and/or efflux phase of the experiment. This is in contradistinction to 10 μM verapamil that must be present in the efflux phase and was maximally active (although still less active than LY335979) when present in both the uptake and efflux phases.

Binding to Pgp. To examine the interaction of LY335979 with Pgp, two binding assays were used. The effect of LY335979 and other modulators was examined on the photoaffinity labeling of Pgp by [^{3}H]azidopine. [^{3}H]Azidopine binds to the M_r 170,000 Pgp protein that is immunoprecipitated with the anti-Pgp monoclonal antibody C219 (8).[3] The effect of LY335979 on photoaffinity labeling was compared to that of verapamil and TMBY at two concentrations, 1 and 5 μM. As shown in Fig. 3, LY335979 was more effective than verapamil or TMBY in reducing [^{3}H]azidopine binding to the M_r 170,000 Pgp protein.

Next, the affinity of Pgp for the modulators was determined using an equilibrium binding assay using radiolabeled oncolytics and plasma membranes prepared from drug-resistant CEM/VLB$_{100}$ cells. Table 5 lists the inhibitory concentration required to displace 50% of bound radiolabeled oncolytic. The IC$_{50}$ to displace 40 nM [^{3}H]vinblastine was determined to be 22.6 μM for verapamil, 3.2 μM for TMBY, and 0.059 μM for LY335979. Similar IC$_{50}$s were obtained when displacement of 40 nM [^{3}H]Taxol and 2 μM [^{3}H]etoposide was examined. To determine the nature of the interaction, a competition assay was conducted by increasing the concentrations of LY335979 in the presence of 40, 100, and 300 nM [^{3}H]vinblastine. A Dixon-Webb plot of these data (Fig. 4) indicated that LY335979 is a competitive inhibitor of [^{3}H]vinblastine with an apparent K_i of 0.059 μM. Taken

[3] R. L. Shepard and A. H. Dantzig, unpublished observations.

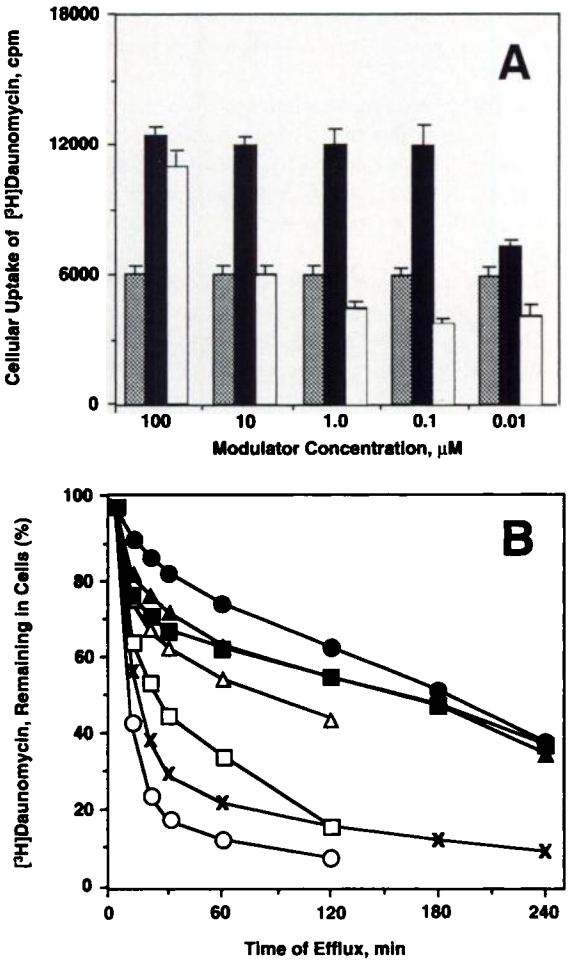

Fig. 2. Effect of modulators on the accumulation and efflux of daunomycin. *A*, effect of verapamil or LY335979 on the accumulation of [^{3}H]daunomycin in 2780AD cells. The tumor cells were incubated for 2.5 h with 0.4 μM [^{3}H]daunomycin in the presence or absence of various concentrations of modulators as described in "Materials and Methods." The data are represented as averages (*bars*, SD) of quadruplicate determinations. ▩, control; ■, LY335979; □, verapamil. *B*, effect of verapamil or LY335979 on the efflux of [^{3}H]daunomycin from 2780AD cells. Cells were incubated for 2 h with 0.3 μM [^{3}H]daunomycin in the presence or absence of the indicated modulator and were subsequently washed and incubated in growth medium for different time periods in the presence or absence of the modulator. The concentrations of verapamil and LY335979 were, respectively, 10 and 0.5 μM. The amount of [^{3}H]daunomycin effluxed from tumor cells was measured as described in "Materials and Methods." Data points represent the average value of quadruplicate determinations. *X*, control; ○, verapamil uptake only; □, verapamil efflux only; △, verapamil uptake and efflux; ●, LY335979 uptake only; ■, LY335979 efflux only; ▲, LY335979 uptake and efflux.

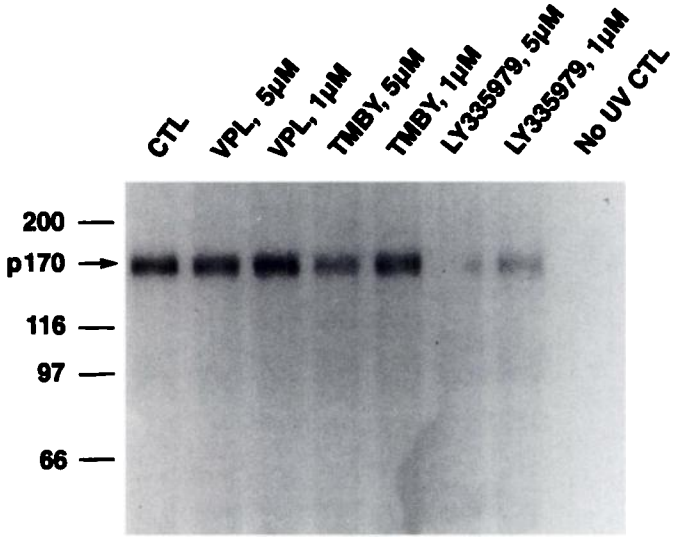

Fig. 3. Fluorograph of 8% SDS-polyacrylamide slab gel of [^{3}H]azidopine photoaffinity labeled proteins of CEM/VLB$_{100}$ plasma membranes. Membranes were incubated with 0.45 μM [^{3}H]azidopine in the presence of 1 or 5 μM of the indicated modulator or in the absence of a modulator (CTL) and irradiated 2 min on ice, as indicated in "Materials and Methods." *Lanes* (*left* to *right*) indicate control (*CTL*), 5 or 1 μM verapamil (*VPL*), 5 or 1 μM TMBY, 5 or 1 μM LY335979, and the control without irradiation (*No UV CTL*). Molecular weight markers are indicated on the *left* (in thousands), and the *arrow* indicates the location of the M_r ~170,000 Pgp protein. In a control experiment, the M_r 170,000 [^{3}H]azidopine-labeled protein was immunoprecipitated from membranes using the Pgp-specific monoclonal antibody C219 and protein A-Sepharose beads (8)[3] and comigrated to the position indicated by the *arrow*.

Table 5 *Affinity of Pgp for modulators*[a]

Drug	IC$_{50}$ (μM)		
	Verapamil	TMBY	LY335979
[^{3}H]Vinblastine	22.6 ± 3.3 (3)	3.2 ± 0.8 (8)	0.060 ± 0.006 (5)
[^{3}H]Taxol	ND[b]	1.1 ± 0.5 (3)	0.030 ± 0.005 (2)
[^{3}H]Etoposide	ND	1.5 (1)	0.060 ± 0.019 (2)

[a] Determined in a binding assay by displacement of the indicated radiolabeled drug. The parentheses indicate the number of independent determinations.
[b] ND, not determined.

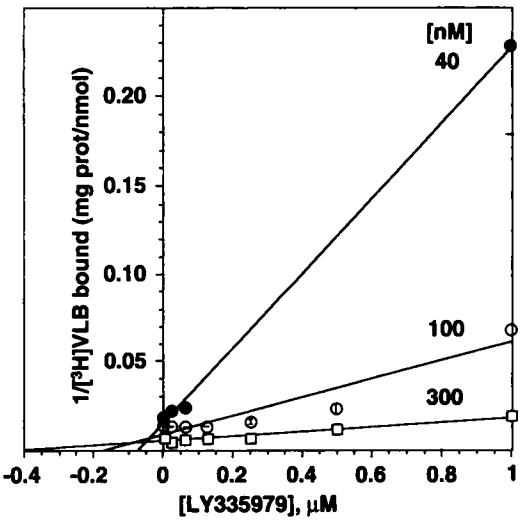

Fig. 4. Dixon-Webb plot of the displacement of [^{3}H]vinblastine binding to CEM/VLB$_{100}$ plasma membranes. Forty nM (●), 100 nM (○), and 300 nM (□) [^{3}H]vinblastine binding was measured in the absence or presence of increasing concentrations of LY335979.

together, these data indicate that Pgp has a very high affinity for LY335979 and that vinblastine and LY335979 share a common binding site.

Effect on Pgp-ATPase Activity. Modulators have been described that stimulate, inhibit, or have no effect on Pgp-ATPase activity (23). Table 6 shows the effect of three modulators on Pgp-ATPase activity. As reported previously, 10 μM verapamil stimulated ATPase activity by 2-fold (24, 25), and 10 μM TMBY inhibited activity slightly. In contrast, LY335979 inhibited Pgp-ATPase activity 50–60% at 2 and 10 μM and had no effect at 0.08 and 0.4 μM. As shown in the cytotoxicity assays, LY335979 modulates well in the 0.08 to 0.4 μM range; therefore, the modulator activity of LY335979 is not dependent on inhibition of the ATPase activity associated with Pgp.

***In Vivo* Efficacy.** The evaluation of LY335979 for *in vivo* efficacy as a reversal agent for Pgp-associated multidrug resistance was performed using the i.p. implanted P388 and P388/ADR murine leukemia cells as well as the s.c. implanted UCLA-P3.003VLB human tumor

Table 6 *Effect on Pgp-ATPase activity*

Drug (μM)	ATPase (μmol/min/mg prot.)	Stimulation (fold)
Control	0.035 ± 0.002	1.00
Verapamil		
(10)	0.071 ± 0.007	2.06
TMBY		
(10)	0.024 ± 0.006	0.70
LY335979		
(10)	0.015 ± 0.011	0.42
(2.0)	0.019 ± 0.010	0.54
(0.4)	0.036 ± 0.002	1.04
(0.08)	0.037 ± 0.001	1.07

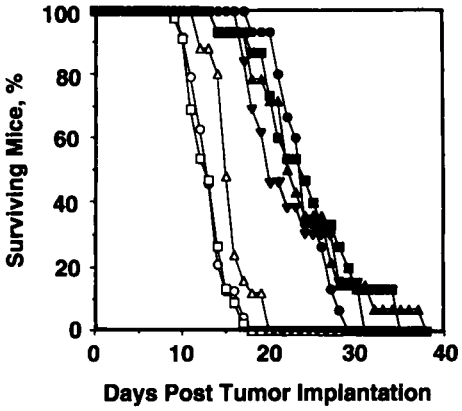

Fig. 5. Effect of i.p. combination therapy of LY335979 and doxorubicin on survival of mice implanted i.p. with P388/ADR murine leukemia cells. Mice were implanted with P388/ADR tumors and treated on days 0–4 with 2 mg/kg doxorubicin (△), 30 mg/kg LY335979 (□), or combinations of 2 mg/kg doxorubicin with 30 mg/kg (●), 10 mg/kg (■), 3 mg/kg (▲), or 1 mg/kg (▼) of LY335979, as described in "Materials and Methods." The graph represents a composite of several controlled experiments and group size ranges from 13–18 mice/treatment group. All of the groups treated with the combination of LY335979 and doxorubicin exhibited a significantly increased survival compared to the group treated with doxorubicin alone ($P < 0.0001$ for all groups). ○, saline.

cell line. Fig. 5 demonstrates that mice implanted with P388/ADR tumors experienced a significant increase in life span when they were treated with a combination of 2 mg/kg doxorubicin plus a wide range of doses (30, 10, 3, or 1 mg/kg) of the LY335979. The specificity of this effect was assessed by comparing the efficacy of combination therapy on mice bearing drug-resistant or drug-sensitive cells (Fig. 6). Again, significant antitumor activity was observed against the MDR P388/ADR cell line when mice were treated with a combined dose of 30 mg/kg LY335979 and 1 mg/kg doxorubicin (Fig. 6*B*), whereas no increased efficacy is observed for the parental P388 cells (Fig. 6*A*) treated with the same drug combination. The survival of mice bearing drug-sensitive tumors treated with doxorubicin plus the modulator LY335979 was not significantly different than mice treated with doxorubicin alone ($P = 0.1$). These data strongly suggest that the potentiation of doxorubicin cytotoxicity by LY335979 *in vivo* is mediated through the interaction of the modulator with Pgp. Since the likely clinical use of LY335979 will use systemic administration of this modulator to patients with tumors that are located distally to the drug injection site, we also examined the ability of this compound to reverse drug resistance when given i.v. The study depicted in Fig. 7 demonstrates that LY335979 can significantly potentiate the antitumor activity of doxorubicin and etoposide when given as a bolus i.v. dose 30 min prior to the i.p. injection of these oncolytics.

The ability of LY335979 to potentiate Taxol activity in a Pgp-expressing human non-small cell lung carcinoma xenograft model is shown in Fig. 8. A combined dose of 20 mg/kg Taxol and 30 mg/kg LY335979 (Fig. 8A) resulted in significant suppression of solid tumor growth at days 12 and 19 compared to either treatment given alone. Importantly, no increased weight loss was observed in any group treated with Taxol plus modulator compared with free drug alone (Fig. 8*B*).

Lack of Effect on Pharmacokinetics. Several Pgp modulators have been reported to alter the pharmacokinetics of oncolytics that are substrates of Pgp and to enhance, therefore, the plasma concentration of the oncolytic (reviewed in ref. 26). Thus, the enhanced life span of mice bearing MDR tumors in the presence of modulator may be due, at least in part, to an increase in drug concentration rather than an enhanced sensitivity of the MDR tumor cells to the oncolytic. To assess whether altered plasma levels of the oncolytic may be responsible for the increased life span observed with LY335979, pharmacokinetic studies (Fig. 9) were conducted with the modulator in combination with either doxorubicin or etoposide in parallel with the *in vivo* studies described above. The effect of LY335979 was compared to that of cyclosporin A and verapamil. A high, efficacious dose of each modulator was used to increase the likelihood of observing an effect in these studies.

The effect of these modulators was examined on the pharmacokinetics of doxorubicin. Mice were treated with a single i.p. dose of 5 mg/kg doxorubicin with or without the i.p. coadministration of 30 mg/kg LY335979, 100 mg/kg cyclosporin A, or 40 mg/kg verapamil. As illustrated in Fig. 9*A*, plasma concentrations of doxorubicin were similar throughout the time course after coadministration of either LY335979 or verapamil compared to doxorubicin alone. The AUC

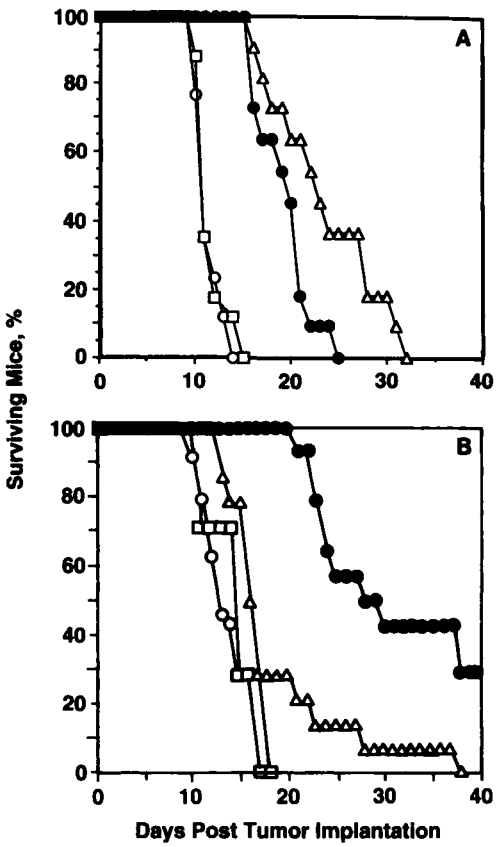

Fig. 6. Effect of i.p. combination therapy of 30 mg/kg LY335979 (□) and 1 mg/kg doxorubicin (△) on i.p. implanted P388 or P388/ADR murine leukemia cells. Mice were implanted with P388 wild-type (*A*) or P388/ADR MDR murine leukemia cells (*B*) and treated on days 0–4 with i.p. administration of doxorubicin, LY335979, or a combination of 30 mg/kg LY335979 and 1 mg/kg doxorubicin (●), as described in "Materials and Methods." The life span of mice implanted with P388 wild-type cells was not significantly different when treated with doxorubicin alone or doxorubicin plus LY335979 ($P = 0.1$; $n = 11$). Treatment of mice implanted with P388/ADR tumor cells with doxorubicin plus LY335979, on the other hand, resulted in a significantly prolonged survival ($P = 0.001$; $n = 7$) relative to mice treated with doxorubicin alone. ○, saline.

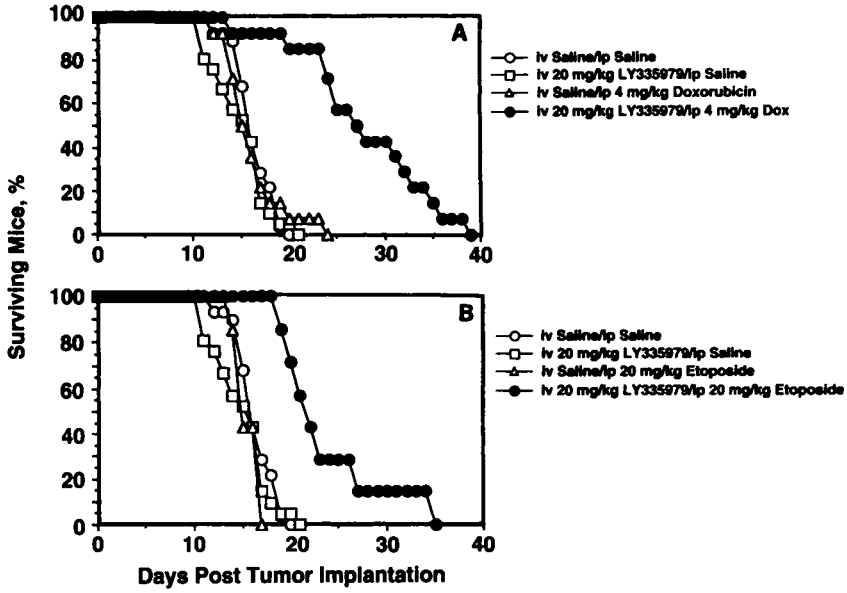

Fig. 7. Effect of i.v. administered LY335979 in combination with doxorubicin or etoposide dosed i.p. on the survival of mice implanted i.p. with P388/ADR murine leukemia tumor cells. Mice were implanted i.p. with P388/ADR tumor cells and treated i.v. with 20 mg/kg LY335979 and i.p. with 4 mg/kg doxorubicin (*A*) or 20 mg/kg etoposide (*B*), as described in "Materials and Methods." The combination therapy with doxorubicin ($P < 0.0001$; $n = 14$) or etoposide ($P = 0.0006$; $n = 7$) plus LY335979 resulted in significantly prolonged survival times compared to treatment with free drug alone.

calculated from these levels, shown in Fig. 9*B*, are quite similar for the control group and those coadministered LY335979 or verapamil. In contrast, doxorubicin levels were higher during the first half of this time period when cyclosporin A was coadministered. Consequently, the resulting AUC with cyclosporin A coadministration was over 200% that of doxorubicin alone and the other two treatment groups and is consistent with previous reports of the effect of cyclosporin A on doxorubicin levels (27, 28). In addition, the i.v. administration of 20 mg/kg LY335979 30 min prior to i.p. injection of 4 mg/kg doxorubicin gave no substantial effect on plasma levels of doxorubicin. In this case, an AUC of 1860 ng·h/ml was measured after a 4 mg/kg i.p. dose of doxorubicin, compared to an AUC of 1430 ng·h/ml with i.v. coadministration of LY335979 (data not shown). Next, the effect of these modulators were examined on the pharmacokinetics of etoposide. Mice were treated with a single administration of etoposide at 30 mg/kg i.p. with or without the i.p. coadministration of 30 mg/kg LY335979, 100 mg/kg cyclosporin A, or 40 mg/kg verapamil; plasma levels are shown in Fig. 9*C* for the time course. Coadministration of all three modulators caused a gradual decline in plasma concentrations (from levels of 600–900 ng/ml measured at 30 min), compared to etoposide alone. At most time points, the levels of etoposide were higher with the coadministration of verapamil and cyclosporin A than with the coadministration of LY335979 or saline. The resulting calculated AUC for each treatment group (Fig. 9*D*) indicated that verapamil and cyclosporin A both caused a dramatic increase in the overall AUC (400–500% greater than the control group as reported previously (29, 30), whereas the effect of LY335979 coadministration was relatively small (about 50% increase in AUC).

DISCUSSION

LY335979 is an extremely potent MDR modulator. *In vitro* full reversal to multidrug resistance can be obtained in the presence of 0.1 μM in several Pgp-expressing cell lines (Tables 1 and 2; Ref. 9). Its ability to modulate at low concentrations is not due to inhibition of ATPase activity associated with Pgp but is due to its ability to inhibit the binding of oncolytics to Pgp. This was demonstrated in both photoaffinity labeling of the M_r 170,000 protein with [^{3}H]azidopine (Fig. 3) and equilibrium binding assays using three different radiolabeled MDR drugs, vinblastine, etoposide, and Taxol (Table 5). The affinity of Pgp for LY335979 is about 0.06 μM when measured with

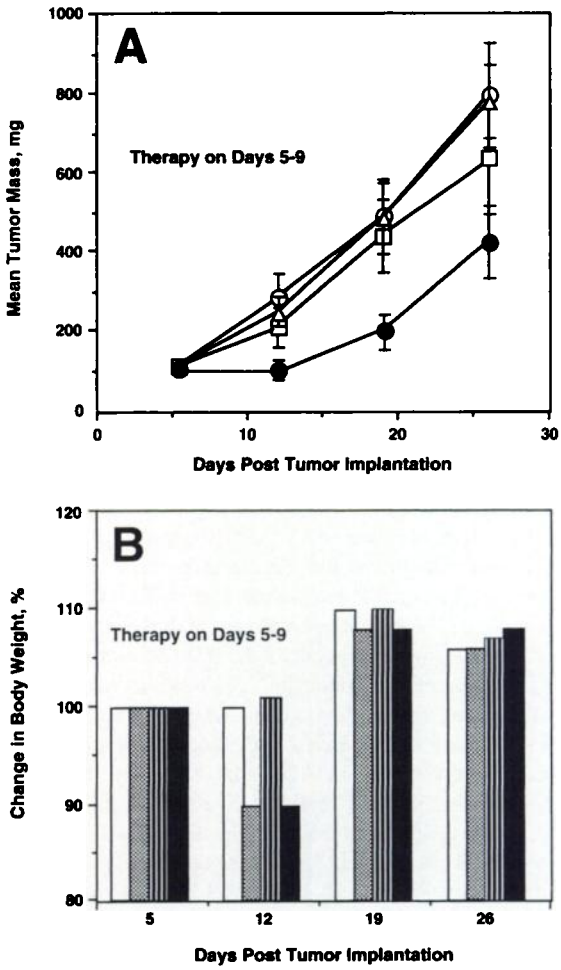

Fig. 8. Effect of Taxol and LY335979 on UCLA-P3.003VLB human non-small cell lung carcinoma xenografts. Nude mice were implanted s.c. with UCLA-P3.003VLB tumor cells and treated with 20 mg/kg Taxol alone or Taxol in combination with 30 mg/kg of LY335979, as described in "Materials and Methods." *A*, the effect of treatment on the increase in tumor mass after tumor implantation. Student's *t* test indicated that treatment with Taxol and LY335979 was significantly different ($P < 0.05$) at days 12 and 19 than any of the other treatments. ○, vehicle; △, 30 mg/kg LY335979; □, 20 mg/kg Taxol; ●, Taxol + LY335979. *B*, the change in body weight for the different treatment groups after tumor implantation. □, vehicle; ▩, 20 mg/kg Taxol; ■, 30 mg/kg LY335979; ■, Taxol + LY335979.

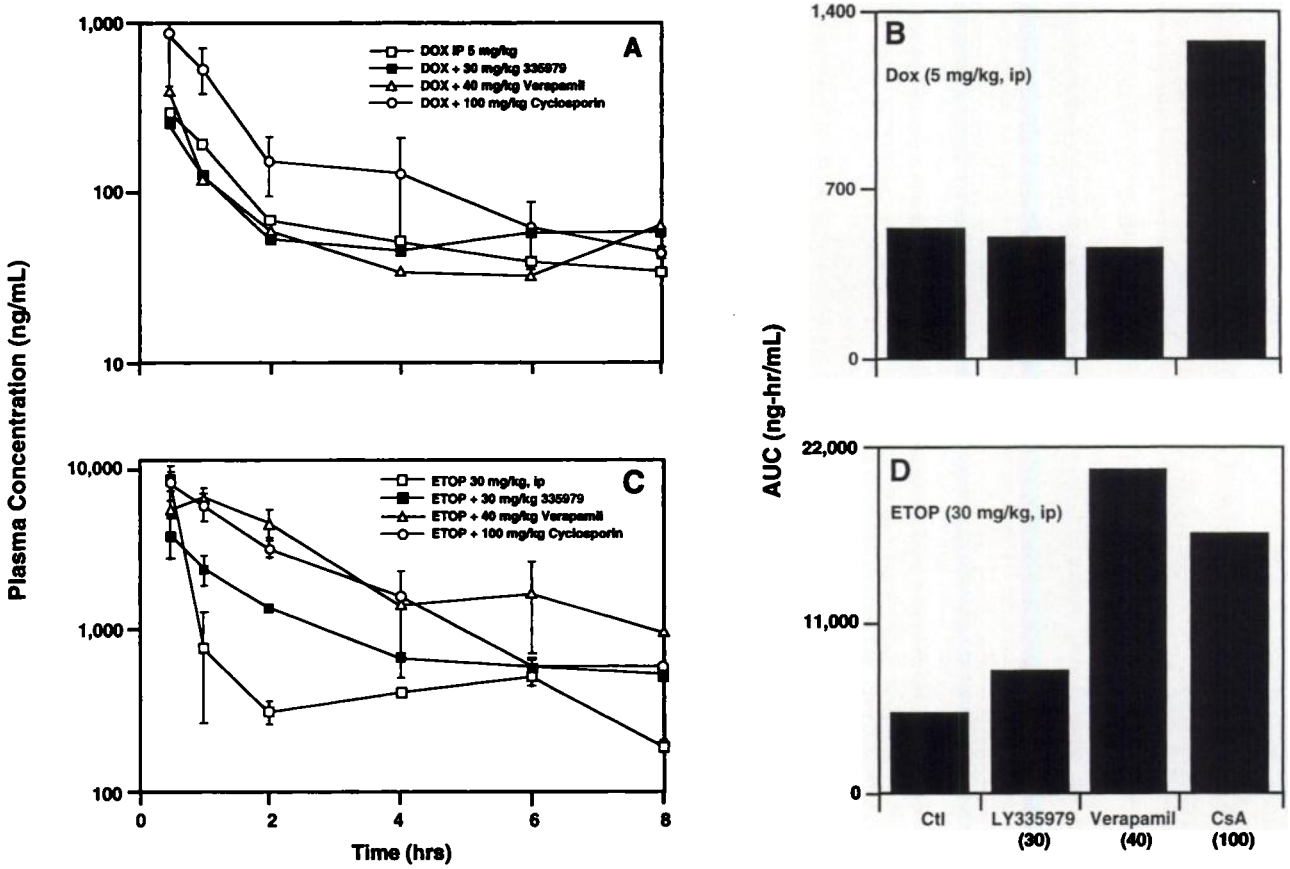

Fig. 9. Effect of coadministration of modulators on plasma concentrations measured over i.p. doses of oncolytic and the resulting AUC. Female BDF_1 mice were administered i.p. either 5 mg/kg doxorubicin (*Dox*, *A* and *B*) or 30 mg/kg etoposide (*Etop*, *C* and *D*) in combination with saline (*Ctl*), 30 mg/kg LY335979, 40 mg/kg verapamil, or 100 mg/kg cyclosporin A (*CsA*). Plasma concentration is shown as a semi-log plot.

different oncolytics. Furthermore, vinblastine and LY335979 share a common binding site, as demonstrated by the competitive binding studies. There was excellent agreement between the affinity of Pgp for the modulator (K_i of 0.059 μM) and the modulator concentration (0.05 μM) needed to give one-half maximal reversal activity to Pgp-expressing cells in cytotoxicity assays. Taken together, these data indicate that the mechanism of action of LY335979 is specific for Pgp and that the compound is a highly potent Pgp modulator.

Studies were conducted to examine the effect of LY335979 on transport by Pgp. The modulator inhibited both the accumulation and the efflux of daunomycin from cells; LY335979 continued to inhibit the efflux of daunomycin from cells, even when it was not present in the efflux medium. This finding is consistent with the observation that the reversal activity of LY335979 is of long duration in doxorubicin cytotoxicity assays with 2780AD cells. Evidently, the compound remains associated with the cell in an active form for prolonged periods of time after removal from the medium prior to the addition of the cytotoxic compound (Table 4). These data, along with the observation that the cytotoxicity of LY335979 by itself is the same for drug-sensitive and drug-resistant cell lines, suggest that LY335979 may not be actively removed from the cell as a substrate of Pgp (Table 3). Thus, LY335979 would be expected to reverse drug resistance over extended periods of time when cells are exposed to drug concentrations at or above the affinity of Pgp.

In vivo, LY335979 reverses Pgp-mediated drug resistance in mice bearing drug-resistant variants of a murine leukemia (Figs. 5, 6, and 7) and a human non-small cell lung carcinoma. Mice bearing P388/ADR cells treated in combination with LY335979 and either doxorubicin or etoposide had increased life spans of about 150%. A wide range of doses of modulator (1–30 mg/kg) were effective in combination therapy without any apparent toxicity to the mice, although the modulator would be expected to alter the normal function of Pgp in normal tissue (Fig. 5). Furthermore, treatment of mice bearing the drug-sensitive P388 parent cell line with 30 mg/kg LY335979 and 1 mg/kg doxorubicin did not enhance their survival time, and no significant increased toxicity was observed (Fig. 6). This clearly indicates that the increase in survival time seen with mice bearing resistant tumors is due to the ability of the drug to block the function of Pgp. Moreover, LY335979 demonstrated a significant enhancement of Taxol efficacy against a MDR human non-small cell lung carcinoma xenograft (Fig. 8) without increasing the toxicity of Taxol to the mice (as judged by body weight loss). Because Taxol is used in the clinical treatment of non-small cell lung carcinoma and Pgp has been shown to be expressed in this tumor type (31, 32), these data would support the use of LY335979 plus Taxol in the treatment of this disease.

Another interesting property of LY335979 is that the modulator had no substantial effect on the pharmacokinetics of both doxorubicin and etoposide in mice at a high dose (30 mg/kg), well above the minimal level (3 mg/kg) necessary for reversal of drug resistance. By contrast, effective doses of both cyclosporin A and verapamil greatly altered the pharmacokinetics of one or both of these drugs (Fig. 9). In Phase 1 trials, the cyclosporin D analogue SDZ PSC 833 has been shown to have a profound effect on the pharmacokinetics of etoposide, even when the dose of the oncolytic was reduced by 50% (33). Whether a Pgp modulator alters pharmacokinetics of the administered oncolytic is likely to be determined by the potency of the modulator for Pgp, the specificity of the modulator for Pgp, and the relative contribution of

Pgp to the pharmacokinetics of the indicated oncolytic. Pharmacokinetic interaction may also occur at sites unrelated to Pgp, such as pathways for drug metabolism, exemplified by the oxidation of doxorubicin by cytochrome P-450 (34–36). Also, the lack of specificity for Pgp has been demonstrated for both cyclosporin A and SDZ PSC 833; they both inhibit a bile acid transporter in the canalicular membrane (37, 38). Consistent with this, the dose-limiting toxicity of cyclosporin A, in combination with doxorubicin or etoposide, clinically is hyperbilirubinemia (37, 39). The apparent lack of pharmacokinetic interaction observed with LY335979 is most similar to GF120918 that also did not alter the pharmacokinetics of doxorubicin (8).

In conclusion, LY335979 is a highly potent Pgp modulator that binds to Pgp and has a long duration of action. It enhances chemotherapeutic activity in MDR-resistant tumors of both hematological and solid tumor origin. The compound apparently does not alter the pharmacokinetics of either doxorubicin or etoposide. It, therefore, represents an exciting new clinical agent to test the hypothesis that inhibition of P-glycoprotein activity will result in reversal of multidrug resistance in both liquid and solid human tumors.

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