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Modulation by LY335979 of P-glycoprotein function in multidrug-resistant cell lines and human natural killer cells

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Abstract

Resistance to chemotherapy by some human tumors may be due to overexpression of membrane-associated transport proteins. The best characterized of these is the multidrug resistance (MDR) transporter, P-glycoprotein (Pgp). The aim of this study was to measure the inhibitory effects of a potent new MDR modulator, (2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl) piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979), in the drug-resistant cell line HL60/VCR and in normal, human $CD56^+$ lymphocytes. We used flow cytometric methods to detect the accumulation of rhodamine 123 and daunorubicin, fluorescent MDR substrates, in these cells. Our results indicate that LY335979 was 500–1500 times more potent than cyclosporin A or verapamil in restoring Pgp substrate accumulation in the MDR cell line HL60/VCR. Moreover, LY335979 could effectively block Pgp function on isolated $CD56^+$ lymphocytes (IC_{50} = 1.2 nM) or $CD56^+$ lymphocytes in whole blood (IC_{50} = 174 nM). We conclude that LY335979 is among the most potent Pgp inhibitors described and that it maintains significant potency in whole-human blood. These latter findings are important for establishing the dosing regimens of LY335979 for future clinical studies.

Keywords: Multidrug resistance; Flow cytometry; P-Glycoprotein; LY335979

1. Introduction

Resistance to chemotherapy by some human tumors is due, in part, to an overexpression of membrane-associated transport proteins. The best characterized of these is the 170 kDa, Pgp. This protein functions as an energy-dependent efflux transporter for a variety of structurally unrelated anti-tumor agents [1–3]. Cells that overexpress Pgp exhibit an MDR phenotype.

A number of substances have been shown to modulate or inhibit the transport function of Pgp, thus enhancing or restoring chemosensitivity of MDR cells to cytotoxic agents. These include VER, CsA, tacrolimus (FK506), yohimbine, and a list of others [4–7]. Unfortunately, attempts to use these agents clinically have met with limited success. A number of new, more potent and selective Pgp inhibitors are in development [8–13]. One such newly developed compound is (2*R*)-anti-5-{3-[4-(10,11-difluoromethano-dibenzo-suber-5-yl) piperazin-1-yl]-2-hydroxy-propoxy} quinoline trihydrochloride (LY335979). This agent has shown an enhanced potency and selectivity in targeting Pgp function both *in vitro* and *in vivo* [8,13]. In published studies, LY335979 enhanced the survival of mice implanted with MDR lung cancer cells that were co-treated with taxol without increasing the toxicity of taxol [13].

Flow cytometry is an invaluable tool for the study of the expression and function of Pgp, as well as the evaluation of putative MDR modulators. The technology has been quite useful for analyses of Pgp function in clinical specimens [14–20]. In the following report, we describe the use of flow cytometry to determine the effects of LY335979 on the efflux activity of MDR-resistant and normal human cells using the fluorescent Pgp substrates, Rh123 and DNR. Our goals were: (a) to reconfirm the potency of LY335979 using a flow cytometric approach, and (b) to develop and validate the activity of LY335979 against Pgp function in normal human blood cells for potential use in subsequent clinical evaluations.

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Abbreviations: MDR, multidrug resistance (resistant); Pgp, P-glycoprotein; Rh123, rhodamine 123; DNR, daunorubicin; TMBY, trimethoxybenzoylyohimbine; CsA, cyclosporin A; VER, verapamil hydrochloride; and MFI, mean fluorescence intensity.

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2. Materials and methods

2.1. Reagents

TMBY and LY335979 (formally known as RS-33295-198, as developed by the Syntex Corp.) were prepared at the Lilly Research Laboratories. CsA and DNR were purchased from the Sigma Chemical Co. Stock solutions (10 mM) of these four 4 compounds were prepared in dimethyl sulfoxide and stored at 4°. Sodium azide and VER (Sigma) were dissolved in RPMI 1640 (No. #22400-089, Life Technologies). Rh123, (Molecular Probes) was prepared as a 1.14 mg/mL stock solution in methanol. Anti-CD56-Cy-Chrome™ antibody was purchased from Pharmingen. CD56 wash buffer was prepared by dissolving sodium azide to 2 mM and bovine serum albumin (No. #82-047-3. Bayer Corp.) to 0.1% (w/v) in calcium/magnesium-free Dulbecco's phosphate-buffered saline (Life Technologies, No. #14190-136).

2.2. Cell lines

The drug-sensitive human myeloblastic leukemia cell line, HL60 (HL60/S), and its drug-resistant variant, HL60/VCR [21], were obtained from Dr. Melvin Center of Kansas State University. The HL60/VCR cell line is 140-fold more resistant to the cytoxic effects of vincristine than its HL60/S parent and strongly expresses Pgp [21]. The two cell lines were cultured in complete growth medium, consisting of RPMI-1640 supplemented with gentamicin (50 μg/mL; Life Technologies, No. #15750-060) and 10% fetal bovine serum (Life Technologies, No. #1600-044). For experiments, the cells were in exponential growth condition and had a density of less than 1.0×10^6 cells/mL.

2.3. Rh123 accumulation in cell lines

HL60/S and HL60/VCR cells were washed twice and resuspended in RPMI-1640 at 1.0×10^6 cells/mL. Aliquots (1 mL) of these cell suspensions were dispensed into 12 × 75 mm polypropylene tubes (Falcon, No. #2063) containing 10 μL of various dilutions (in RPMI) of either LY335979, TMBY, CsA, VER, or diluent alone and were incubated at 37° for 1 hr. Rh123 was then added to the samples as 10 μL of a 100× solution (final concentration 150 ng/mL), and tubes were incubated at 37° for defined intervals. After this incubation, the tubes were kept on ice until analysis on the flow cytometer.

2.4. Rh-123 accumulation in $CD56^+$ lymphocytes

Venous blood from normal human volunteers, who gave informed, written consent, was collected into Becton Dickinson Vacutainer® CPT™ tubes (No. #362753, Becton Dickinson Vacutainer Systems). These evacuated blood collection tubes (8 mL draw) contained sodium heparin anticoagulant in the upper section of the tube and a Ficoll-Hypaque density gradient liquid in the bottom, separated by a layer of polyester gel. In some experiments, LY335979 (or RPMI diluent) was added to the collected whole blood specimen, mixed well, and incubated at room temperature (RT) for 1 hr. Tubes were then centrifuged for 20 min at 1500 *g* at RT, per instructions of the manufacturer. After centrifugation, the resultant mononuclear cells and platelets (residing above the gel barrier) were resuspended in the plasma layer by inverting the tubes 3–4 times, and then the tubes were stored overnight at 4°. The following morning, the mononuclear cell/plasma layer from each CPT™ tube was decanted into a clean 50-mL polypropylene centrifuge tube (Falcon, No. #2098), 40 mL RPMI was added, and the sample was centrifuged at 150 *g* for 10 min at 4°. The supernatant was removed, the wash step was repeated, and the cells were resuspended in RPMI at 1×10^{-6}/mL. Aliquots (990 μL) of cell suspension were dispensed into 12 × 75 mm polypropylene tubes containing 10 μL of an LY335979 dilution (in RPMI) or diluent alone and incubated at 37° for 30 min. Rh123 was added at a final concentration of 50 ng/mL, and the tubes were incubated-further at 37° for defined intervals. Next, 3 mL of ice-cold CD56 wash buffer was added. Samples were centrifuged for 5 min at 400 *g* at 4°. All but approximately 80 μL of supernatant was discarded, and 20 μL of anti-CD56 antibody (neat) was added, mixed gently, and incubated at 37° for 15 min. To lyse contaminating red cells, 1 mL of ice-cold distilled water was added to the tubes, and vortexed, and after exactly 30 sec, 1 mL of ice-cold, 2× phosphate-buffered saline was added vigorously. Samples were centrifuged for 5 min at 400 *g* at 4°, the supernatant was discarded, and the samples were resuspended in 750 μL of ice-cold CD56 wash buffer and kept on ice. Each set of assay tubes included single color controls to adjust for spectral overlap in the subsequent two-color fluorescence analysis.

2.5. Daunorubicin efflux

Two aliquots, 10 million cells each, from actively growing cultures of HL60/S and HL60/VCR were placed in 50-mL tubes (Falcon, No. #2098) and washed once in glucose-free RPMI (Life Technologies, No. #11879-020). Cells were resuspended in glucose-free RPMI containing 10 mM NaN_3 and DNR (1.0 μM), vortexed, and placed in a 37° water bath for 2 hr. This procedure effectively loaded the fluorescent Pgp substrate, DNR, by preventing efflux. Following this incubation, cells were washed twice with ice-cold glucose-free RPMI and resuspended in either 5 mL of ice-cold complete growth medium (RPMI with glucose and 10% fetal bovine serum) or ice-cold complete growth medium containing 500 nM LY335979. A 2.0-mL aliquot of each was immediately measured on the flow cytometer for DNR, and then remaining cell mixtures were transferred

to a 37° water bath and re-sampled for DNR fluorescence every 5 min for an additional 30 min or at 90 min.

2.6. Flow cytometry

Reacted samples were analyzed for light scatter and fluorescence using an Epics XL flow cytometer (Beckman-Coulter Corp.) equipped with a 488 nm air-cooled argon ion laser. For cell culture samples, 10,000 cells were analyzed for forward and 90 degree light scatter and either Rh123 (green) fluorescence or DNR (red) fluorescence through a 525 or 620 nm bandpass filter, respectively. For human blood samples, data for light scatter and Rh123 fluorescence were collected as above for 30,000 cells from each sample. In addition, Cy-Chrome™ (far-red) fluorescence of blood cell samples was collected through a 630 nm long pass filter, and subsequently used to identify $CD56^+$ cells. No hardware color compensation was used during data acquisition. All fluorescence data were acquired on a logarithmic scale and stored in listmode files.

2.7. Data analysis

For the treated cell line samples, the light scatter-gated (to include viable, single cells) MFI for Rh123 or DNR of each sample was determined for each listmode data file using WinList™ software (Verity Software House). For the treated human blood samples, two-color fluorescence analysis of the listmode files was performed in order to determine the Rh123 fluorescence associated with the $CD56^+$ lymphocytes contained within the mixed mononuclear cell samples. Samples stained with either Rh123 or anti-CD56-Cy-Chrome™ antibody alone were first analyzed in order to set color compensation for spectral overlap of the resultant green and far-red fluorescence emissions, using an included algorithm of WinList™ software. The remaining samples of each set were then analyzed for the MFI of the Rh123 (green fluorescence) associated with a $CD56^+$ (far-red fluorescence) gate.

2.8. Calculations

For some Rh123 experiments with HL60/S and H60/VCR cells, compound activity was expressed as "% inhibition". This was calculated according to the formula:

$$\%\ \text{inhibition} = 100 \times \{(\text{MFI}_{\text{test}} - \text{MFI}_{\text{control}})/(\text{MFI}_{\text{max}} - \text{MFI}_{\text{control}})\}$$

where MFI_{test} represents mean Rh123 fluorescence intensity for the treated sample; $\text{MFI}_{\text{control}}$, the fluorescence of the diluent only sample; and MFI_{max}, the maximal fluorescence observed over the entire sample set. The concentration of compound that achieved 50% inhibition of Rh123 efflux (IC_{50}) was determined from a regression analysis of the % inhibition versus (log) compound concentration using a sigmoidal dose-response curve fit of Prism™ software, (Graph-Pad).

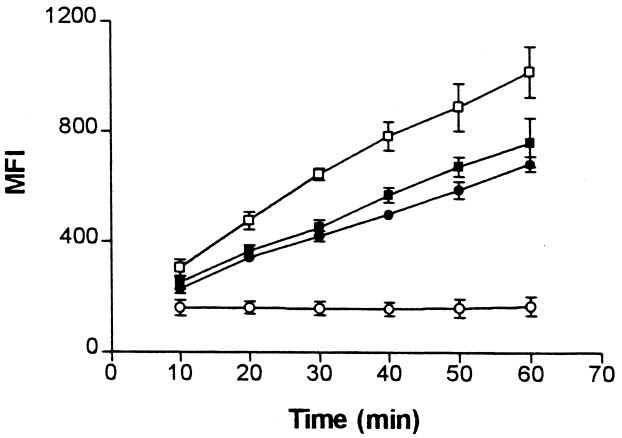

Fig. 1. Accumulation of Rh123 by HL60/S and HL60/VCR cell lines. Cells were washed, resuspended in RPMI culture medium or LY335979 (1 μM final concentration), and incubated for 60 min at 37°. Rh123 (150 ng/mL) was then added to all tubes, and the tubes were incubated further at 37°. At the time points shown above, the samples were analyzed by flow cytometry. Each point represents the average MFI ± SEM for three separate experiments. Key: (□) HL60/S with buffer, (■) HL60/S with LY335979, (○) HL60/VCR with buffer, and (●) HL60/VCR with LY335979.

3. Results

Effect of LY335979 on Rh123 uptake by HL60 cells

The functional activity of Pgp was measured using Rh123, a fluorescent Pgp substrate. We used drug-sensitive (HL60/S) and -resistant (HL60/VCR) variants of the HL60 cell line to study the effect of LY335979 on Rh123 accumulation. As shown in Fig. 1, when HL60/S cells were incubated with Rh123. Rh123 fluorescence increased in a linear manner over a 1-hr period, to about 2.5 times over the starting level. In contrast, untreated HL60/VCR did not accumulate substrate over this same time period. When the same cell line (HL60/VCR) was pre-incubated with LY335979 (1 μM), it accumulated Rh123 in a manner similar to that of the untreated HL60/S cells. No such increase in Rh123-associated fluorescence was seen with LY335979-treated HL60/S cells.

3.2. Comparison of the activity of LY335979 with the activities of other Pgp modulators

Using the same assay of Pgp function with HL60/VCR cells, we compared the effects of LY335979 with that of three other Pgp modulators as a function of compound concentration. Fluorescence data obtained from these studies were normalized as described in "Materials and methods". Data from these experiments, shown in Fig. 2, yielded the following IC_{50} values: LY335979, 6.74 nM; CsA, 3.78

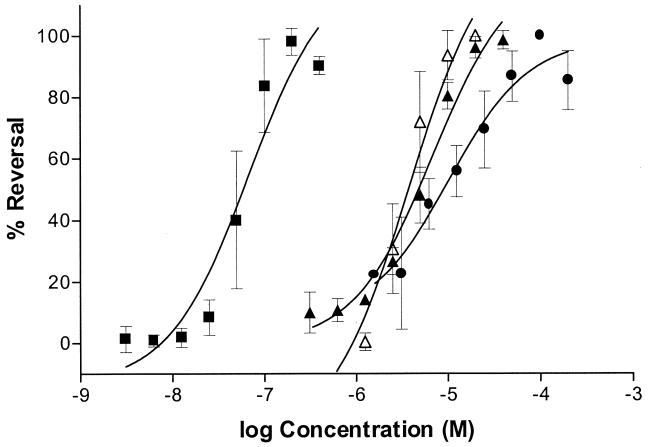

Fig. 2. Effects of LY335979, CsA, TMBY, or VER, on uptake of Rh123 by HL60/VCR cells. Cells were washed and resuspended in RPMI culture medium or compounds at concentrations shown in the fig. After a 60-min incubation at 37°, Rh123 (150 ng/mL) was added to all samples, and the cells were incubated for another 60 min at 37°. The samples were then analyzed by flow cytometry, and the percent reversal was computed as described in the text. The data points represent the average percent reversal ± SEM for three separate experiments. The curves were fit using a sigmoidal dose-response algorithm. Key: (■) LY335979 (IC_{40} = 6.74 nM), (△) CsA (IC_{50} = 3.78 μM), (▲) TMBY (IC_{50} = 6.96 μM), and (●) VER (IC_{50} = 10.34 μM).

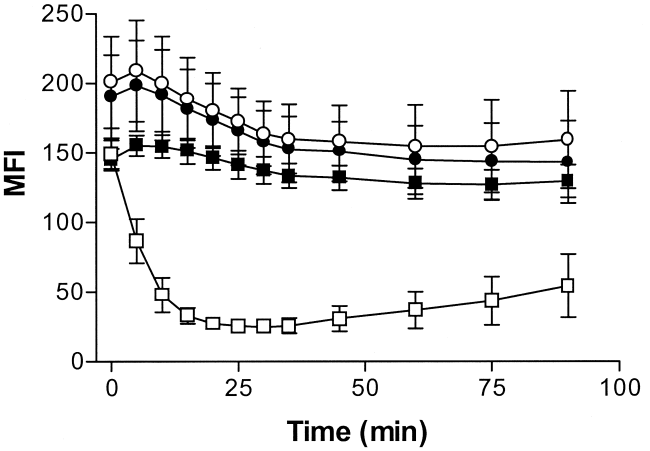

Fig. 3. Effect of LY335979 on retention of DNR in HL60/S and HL60/VCR cells. HL60/S and HL60/VCR cells were loaded with DNR (1 μM) in glucose-free RPMI medium containing sodium azide (10 mM) for 2 hr at 37°. Cells were then washed and resuspended in glucose-containing RPMI ± LY335979 (500 nM) and incubated at 37° for the times indicated. At each time point, the DNR-associated fluorescence was measured using flow cytometry. Key: (□) HL60/VCR with buffer, (■) HL60/VCR with LY335979, (○) HL60/S with buffer, and (●) HL60/S with LY335979. Values are averages ± SEM for three experiments.

μM; TMBY, 6.96 μM; and VER, 10.34 μM. These data indicate that LY335979 is approximately 500 times more potent than CsA and 1500 times more potent than VER in blocking the Pgp activity of HL60/VCR cells.

3.3. Blockade of DNR efflux by LY335979

In the next experiments, we loaded HL60 cells with DNR and studied the effects of LY335979 on the efflux of this fluorescent Pgp substrate. During the loading process, we blocked energy-dependent Pgp function by removing glucose and adding sodium azide. As displayed in Fig. 3, HL60/VCR cells rapidly effluxed loaded DNR after the azide was removed and glucose was added back into the growth medium. By 20 min, 80% of the DNR-associated fluorescence was gone from the HL60/VCR cells. If, however, LY335979 (500 nM) was present in the efflux medium, little or no change in DNR fluorescence was observed. LY335979 was effective in blocking DNR efflux from HL60/VCR cells down to a concentration of 50 nM (data not shown). Note that LY335979 efflux blockade of HL60/VCR cells occurred without pre-exposure of cells to inhibitor. In contrast, LY335979 had little effect on HL60/S cells. In addition, we compared the potency of LY335979 in the DNR retention assay to that of CsA and VER in three experiments. LY335979 (IC_{50} = 20 ± 2.9 nM, mean ± SEM) was, on average, 75-fold more potent than CsA (IC_{50} = 1.5 ± 0.37 μM) and 120-fold more potent than VER (IC_{50} = 2.4 ± 0.6 μM) in blocking DNR efflux by HL60/VCR cells (data not shown).

3.4. Effect of LY335979 on Rh123 accumulation in isolated human lymphocytes

Normal human natural killer (NK) cells display Pgp function [22,23]. In Fig. 4 we show how this activity was observed in a two-color flow cytometric analysis. In this assay, the NK cells are defined by their positive reactivity to anti-CD56 antibody (*y*-axis) and by Pgp function as determined by Rh123 accumulation (*x*-axis). Using this approach, the Rh123 fluorescence associated with the $CD56^+$ population was determined. In this representative experiment, an increase in NK cell-associated Rh123 fluorescence was observed after treatment with LY335979. The mean Rh123 fluorescence of the $CD56^+$ cells increased 14.8-fold while that of the remaining cells by only 2.9-fold. As shown in Fig. 5, this effect of LY335979 on $CD56^+$ cell function was concentration dependent. The IC_{50} for LY335979 on isolated $CD56^+$ cells was determined to be 1.18 ± 0.2 nM.

3.5. Effect of LY335979 on Rh123 accumulation in human lymphocytes suspended in whole blood

The addition of LY335979 to whole, human blood also blocked the efflux function of $CD56^+$ lymphocytes. As shown in Fig. 6, the Rh123-associated, cellular fluorescence was proportional to the concentration of LY335979 added to the whole blood sample. The IC_{50} of LY335979 for $CD56^+$ cells residing in whole blood was 174 ± 1.61 nM or about 150 times less potent than that observed on the isolated lymphocyte cell preparations.

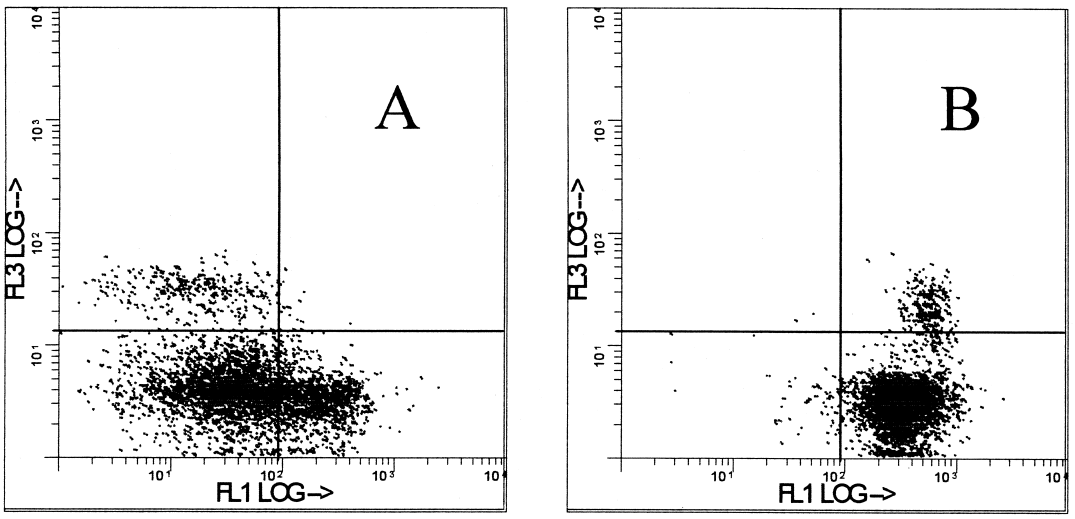

Fig. 4. Effect of LY335979 on Rh123 accumulation in normal CD56$^+$ lymphocytes. A mononuclear cell fraction was obtained from a normal human blood sample by density gradient separation. The cells were then incubated with Rh123 (50 ng/mL) for 90 min at 37° and incubated further with anti-CD56-Cy-Chrome™ antibody to detect the natural killer cell subpopulation. Next the samples were analyzed using flow cytometry for both green (FL1 Log; Rh123) and red (FL3 Log; Cy-Chrome™) fluorescence. Panel A shows untreated mononuclear cells, and panel B depicts mononuclear cells treated with LY335959 (1 μM).

4. Discussion

Several lines of evidence suggest the importance of Pgp activity in chemotherapy resistance for some human cancers [1,3,19,24,25]. In this regard, new potent and selective Pgp inhibitors have entered clinical trials. One of these agents, LY335979, has been tested in cell lines and animal models and is a potent, selective Pgp inhibitor [11,13]. Furthermore, unlike a number of other investigational MDR modulators, [26–28], in preclinical studies LY335979 did not alter the pharmacokinetics of co-administered cytotoxic agents [13].

Flow cytometry has proven to be a powerful tool for studying the MDR phenotype and the effects of Pgp inhibitors. Rh123, a fluorescent substrate, has been shown to be a sensitive reagent for the detection of Pgp function [19,29]. The type of multiparametric analysis made possible by flow cytometric technology is highly informative for understand-

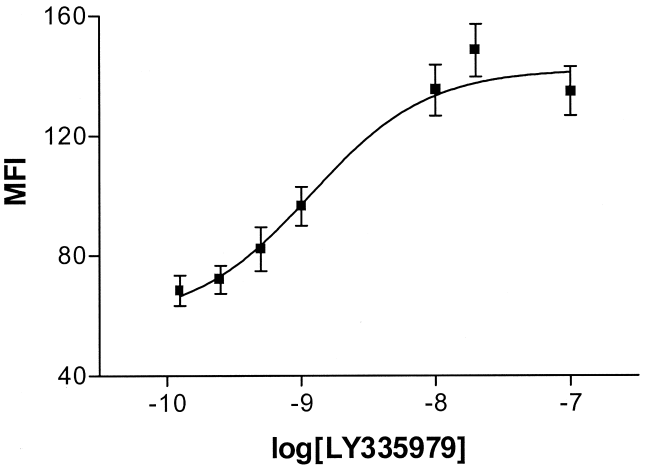

Fig. 5. Effect of LY335979 on the accumulation of Rh123 by isolated CD56$^+$ lymphocytes. Isolated mononuclear cells were incubated with LY335979 at the concentrations (M) shown for 90 min at 37°. The cells were then further incubated with Rh123 for 1 hr and analyzed using flow cytometry. Each point represents the mean ± SEM for four separate human donors. The data points were fit to a sigmoidal dose-response curve using GraphPad Prism™ software.

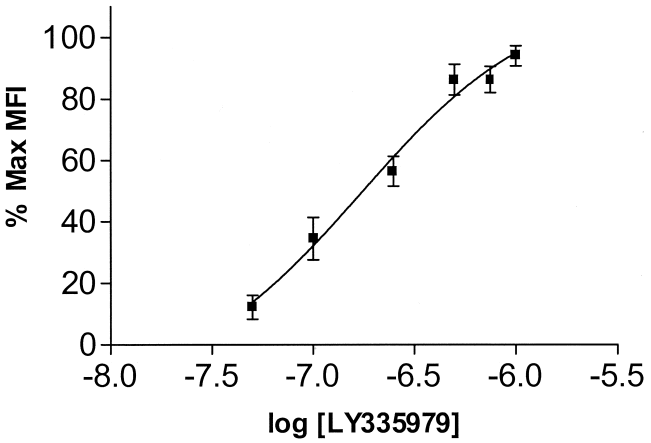

Fig. 6. Effect of LY335979 on Rh123 accumulation in CD56$^+$ lymphocytes in whole human blood. Normal human blood was drawn into Vacutainer® CPT™ tubes and incubated with various concentrations (M) of LY335979 for 1 hr at room temperature. The tubes were then centrifuged and stored at 4° overnight. The following morning, the mononuclear cell layer was removed, washed, and incubated with Rh123 (50 ng/mL) for 90 min at 37°. The samples were then stained with anti-CD56-Cy-Chrome™ antibody, and analyzed by flow cytometry. Each data point represents the mean ± SEM of eight human donors. The data were curve fit as described in the text.

ing the prevalence and prognostic significance of Pgp-associated human MDR. Consensus recommendations for the detection of Pgp expression and activity in human tumors advised the use of flow cytometry for detection of Pgp activity [18].

In the first series of experiments, we measured the effects of LY335979 on Rh123 accumulation in a human cell line that overexpresses Pgp. Results from these studies confirmed that LY335979 is among the most potent Pgp inhibitors that have been described. LY335979 was approximately 500 times more potent than CsA and 1500 times more potent than VER in increasing Rh123 accumulation (Fig. 2). In the studies with DNR (Fig. 3), we confirmed that the mechanism of LY335979 activity included inhibition of energy-dependent efflux.

We also measured the effects of LY335979 on Rh123 efflux from normal, human $CD56^+$ lymphocytes. The $CD56^+$ lymphocyte population has been shown to reliably express functional Pgp on cell membrane surfaces [22,23, 30–32]. The inhibitory activity on these cells was approximately 5-fold more potent (IC_{50} = 1.18 vs 6.74 nM) than that observed with HL60/VCR cells. Cell surface expression of Pgp on normal $CD56^+$ lymphocytes is somewhat lower than that observed on highly drug-resistant, laboratory-generated cell lines [23,32] and may more closely resemble that of human tumor cells.

Additionally, we characterized the potency of LY335979 in whole blood. In these experiments (Fig. 6), LY335979 bound to $CD56^+$ cell Pgp in the presence of the plasma proteins and cellular elements. Significant inhibition of Pgp function was observed, even after extensive washing. This result is consistent with previous reports that LY335979 could sensitize cells to doxorubicin even after compound removal [13]. In these studies, we simulated patient specimens by adding known amounts of LY335979 to whole blood samples in CPT™ collection tubes. Our IC_{50} calculation of 174 nM is based upon the initial LY335979 added to whole blood. In a prior study (unpublished data), LY335979 was shown to be 70% protein bound. Decreased potency in the presence of plasma proteins has been reported for other MDR modulators such as GF120918 and SDZ PSC 833 [33,34]. Our data provides an estimate of the required plasma concentration of LY335979 for Pgp inhibition in human $CD56^+$ lymphocytes *in vivo*.

Our ability to measure LY335979 inhibition of $CD56^+$ lymphocyte Pgp function is important because other investigators have shown that these human cells provide excellent surrogate targets in demonstrating pharmacological activity of MDR modulators during early clinical trials [20, 35]. This surrogate efficacy assay could, if incorporated in first human dose studies, aid the selection of an appropriate dose to test in larger efficacy trials. The data presented here suggest that our assays may prove informative and could enhance the clinical development of this MDR modulator.

References

[1] Kartner N, Ling V. Multidrug resistance in cancer. Sci Am 1989;260: 44–51.

[2] Krishan A, Fitz CM, Andritsch I. Drug retention, efflux, and resistance in tumor cells. Cytometry 1997;29:279–85.

[3] Gottesman MM. How cancer cells evade chemotherapy: Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 1993;53:747–54.

[4] Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S, Aszalos A. The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multi-drug transporter. Int J Cancer 1993;54:456–61.

[5] Ross DD, Wooten PJ, Sridhara R, Ordonez JV, Lee EJ, Schiffer CA. Enhancement of daunorubicin accumulation, retention, and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukemia. Blood 1993;82:1288–99.

[6] Sonneveld P, Wiemer E. Inhibitors of multidrug resistance. Curr Opin Oncol 1997;9:543–8.

[7] Ford JM. Experimental reversal of P-glycoprotein-mediated multidrug resistance by pharmacological chemosensitisers. Eur J Cancer 1996;32A:991–1001.

[8] Slate DL, Bruno NA, Casey SM, Zutshi N, Garvin LJ, Wu H, Pfister RS. 33295-198: a novel, potent modulator of P-glycoprotein-mediated multidrug resistance. Anticancer Res 1995;15:811–4.

[9] Starling JJ, Shepard RL, Cao J, Law KL, Norman BH, Kroin JS, Ehlhardt WJ, Baughman TM, Winter MA, Bell MG, Shin C, Gruber J, Elmquist WF, Dantzig AH. Pharmacological characterization of LY335979: a potent cyclopropyldibenzosuberane modulator of P-glycoprotein. Adv Enzyme Regul 1997;37:335–47.

[10] Mistry P, Plumb J, Eccles S, Watson S, Dale I, Ryder H, Box G, Charlton P, Templeton D, Bevan PB. *In vivo* efficacy of XR9051, a potent modulator of P-glycoprotein mediated multidrug resistance. Br J Cancer 1999;79:1672–8.

[11] Dantzig AH, Shepard RL, Law KL, Tabas L, Pratt S, Gillespie JS, Binkley SN, Kuhfeld MT, Starling JJ, Wrighton SA. Selectivity of the multidrug resistance modulator, LY335979, for P-glycoprotein and effect on cytochrome P-450 activities. J Pharmacol Exp Ther 1999; 290:854–62.

[12] Germann UA, Shlyakhter D, Mason VS, Zeile RE, Duffy JP, Galullo V, Armistead DM, Saunders JO, Boger J, Harding MW. Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance *in vitro*. Anticancer Drugs 1997;8:125–40.

[13] Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM, Bumol TF, Starling JJ. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res 1996;56:4171–9.

[14] Tatu CA, Paunescu V, Stanescu DL, Schneider F. P-glycoprotein mediated multidrug resistance assessment by flow-cytometry in malignant hemopathies. Anticancer Res 1997;17:2583–6.

[15] Feller N, Kuiper CM, Lankelma J, Ruhdal JK, Scheper RJ, Pinedo HM, Broxterman HJ. Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry [published erratum appears in Br J Cancer 1996;74:2042]. Br J Cancer 1995;72:543–9.

[16] Krishan A, Sauerteig A, Andritsch I, Wellham L. Flow cytometric analysis of the multiple drug resistance phenotype. Leukemia 1997: 11:138–46.

[17] Petriz J, Garcia-Lopez J. Flow cytometric analysis of P-glycoprotein function using rhodamine 123. Leukemia 1997;11:1124–30.

[18] Beck WT, Grogan TM, Willman CL, Cordon-Cardo C, Parham DM, Kuttesch JF, Andreeff M, Bates SE, Berard CW, Boyett JM, Brophy NA, Broxterman HJ, Chan HS, Dalton WS, Dietel M, Fojo AT, Gascoyne RD, Head D, Houghton PJ, Srivastava DK, Lehnert M, Leith CP, Paietta E, Pavelic ZP, Weinstein R. Methods to detect

P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. Cancer Res 1996;56:3010–20.
[19] Broxterman HJ, Schuurhuis GJ. Transport proteins in drug resistance: detection and prognostic significance in acute myeloid leukemia. J Intern Med Suppl 1997;740:147–51.
[20] Witherspoon SM, Emerson DL, Kerr BM, Lloyd TM, Dalton WS, Wissel PS. Flow cytometric assay of modulation of P-glycoprotein function in whole blood by the multidrug resistance inhibitor GG918. Clin Cancer Res 1996;2:7–12.
[21] McGrath T, Latoud C, Arnold ST, Safa AR, Felsted RL, Center MS. Mechanisms of multidrug resistance in HL60 cells. Analysis of resistance associated membrane proteins and levels of *mdr* gene expression. Biochemical Pharmacology 1989;38:3611–9.
[22] Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. Blood 1992;80:2735–9.
[23] Klimecki WT, Futscher BW, Grogan TM, Dalton WS. P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood 1994;83:2451–34.
[24] te Boekhorst PAW, de Leeuw K, Schoester M, Wittebol S, Nooter K, Hagemeijer A, Loowenberg B, Sonneveld P. Predominance of functional multidrug resistance (MDR-1) phenotype in $CD34^+$ acute myeloid leukemia cells. Blood 1993;82:3157–62.
[25] Sonneveld P, Schoester M, de Leeuw K. Clinical modulation of multidrug resistance in multiple myeloma: effect of cyclosporine on resistant tumor cells. J Clin Oncol 1994;12:1584–91.
[26] Bartlett NL, Lum BL, Fisher GA, Brophy NA, Ehsan MN, Halsey J, Sikic BLI. Phase I trial of doxorubicin with cyclosporine as a modulator of multidrug resistance. J Clin Oncol 1994;12:835–42.
[27] Boote DJ, Dennis IF, Twentyman PR, Osborne RJ, Laburte C, Hensel S, Smyth JF, Brampton MH, Bleehen NM. Phase I study of etoposide with SDZ PSC 833 as a modulator ofmultidrug resistance in patients with cancer. J Clinr Oncol 1996;4:610–48.
[28] Lum BL, Fisher GA, Brophy NA, Yahanda AM, Adler KM, Kaubisch S, Halsey J, Sikic BI. Clinical trials of modulation of multidrug resistance. Pharmacokinetic and pharmacodynamic considerations. Cancer 1993;72:3502–14.
[29] Broxterman HJ, Sonneveld P, Feller N, Ossenkoppele GJ, Wahrer DC, Eekman CA, Schoester M, Lankelma J, Pinedo HM, Lowenberg B, Schuurhuis GJ. Quality control of multidrug resistance assays in adult acute leukemia: correlation between assays for P-glycoprotein expression and activity. Blood 1996;87:4809–16.
[30] Lum BL, Gosland MP. MDR expression in normal tissues. Pharmacologic implications for the clinical use of P-glycoprotein inhibitors. Hematol Oncol Clin North Am 1995;9:319–36.
[31] Drach D, Zhao S, Drach J, Mahadevia R, Gattringer C, Huber H, Andreeff M. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. Blood 1992;80:2729–34.
[32] Coon JS, Wang YZ, Bines SD, Markham PN, Chong AS, Gebel HM. Multidrug resistance activity in human lymphocytes. Hum Immunol 1991;32:134–40.
[33] Petriz J, Sanchez J, Bertran J, Garcia-Lopez J. Comparative effect of verapamil, cyclosporin A and SDZ PSC 833 on rhodamine 123 transport and cell cycle in vinblastine-resistant Chinese hamster ovary cells overexpressing P-glycoprotein. Anticancer Drugs 1997;8:869–75.
[34] den Ouden D, van den Heuvel M, Schoester M, van Rens G, Sonneveld P. *In vitro* effect of GF120918, a novel reversal agent of multidrug resistance, on acute leukemia and multiple myeloma cells. Leukemia 1996;10:1930–6.
[35] Robey R, Bakke S, Stein W, Meadows B, Litman T, Patil S, Smith T, Fojo T, Bates S. Efflux of rhodamine from $CD56^+$ cells as a surrogate marker for reversal of P-glycoprotein-mediated drug efflux by PSC 833. Blood 1999;93:306–14.