HG-829 Is a Potent Noncompetitive Inhibitor of the ATP-Binding Cassette Multidrug Resistance Transporter ABCB1

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Abstract

Transmembrane drug export mediated by the ATP-binding cassette (ABC) transporter P-glycoprotein contributes to clinical resistance to antineoplastics. In this study, we identified the substituted quinoline HG-829 as a novel, noncompetitive, and potent P-glycoprotein inhibitor that overcomes in vitro and in vivo drug resistance. We found that nontoxic concentrations of HG-829 restored sensitivity to P-glycoprotein oncolytic substrates. In ABCB1-overexpressing cell lines, HG-829 significantly enhanced cytotoxicity to daunorubicin, paclitaxel, vinblastine, vincristine, and etoposide. Coadministration of HG-829 fully restored in vivo antitumor activity of daunorubicin in mice without added toxicity. Functional assays showed that HG-829 is not a Pgp substrate or competitive inhibitor of Pgp-mediated drug efflux but rather acts as a noncompetitive modulator of P-glycoprotein transport function. Taken together, our findings indicate that HG-829 is a potent, long-acting, and noncompetitive modulator of P-glycoprotein export function that may offer therapeutic promise for multidrug-resistant malignancies. Cancer Res; 72(16); 1–10. ©2012 AACR.

Introduction

De novo or acquired multidrug resistance (MDR) arising from intrinsic cytoprotective mechanisms or tumor cell interaction with the microenvironment remains a major obstacle to successful cancer treatment. The ATP-binding cassette (ABC) transporters form a superfamily of transmembrane proteins that export a wide variety of substrates that range from ions, amino acids, and lipids to oligopeptides and drugs (1, 2). Included among the latter are amphiphatic antineoplastics such as anthracyclines, vinca alkaloids, taxanes, and topoisomerase inhibitors (2). Transfection of ABC transporters is alone sufficient for in vitro drug resistance, and in the setting of corresponding gene overexpression in primary malignancies, such transport proteins have been implicated in clinical chemotherapy resistance. The primary members of the ABC transporters linked to clinical MDR, as reviewed by Szakacs and colleagues (3), include ABCB1 [P-glycoprotein (Pgp), MDR-1], ABCCs (MRP), and ABCG2 (BCRP/MXR/ABCP). A large number of compounds, including cyclosporines, calmodulin inhibitors, and carboximide derivatives, and others have been identified to inhibit one or more of these transporters to suppress drug efflux and restore cellular cytotoxicity (2). The vast majority of efflux modulators serve as competitive substrate inhibitors. Substrate binding to Pgp recruits ATP, which is believed to initiate dimerization in the nucleotide-binding site with corresponding structural changes favoring an outward-facing conformation facilitating drug export (4). The recent discovery of at least 4 distinct drug interaction sites for Pgp suggests that effective suppression of transmembrane export by such inhibitors will be substrate dependent (5–7).

With the exception of cyclosporin A in high-risk acute myeloid leukemia, clinical trials testing first-generation Pgp inhibitors were disappointing (8). The second generation of Pgp-specific modulators such as valspodar (PSC833) had greater potency but deleterious pharmacokinetic interactions and unsatisfactory outcomes in phase III trials (9–12). Initial studies of third-generation Pgp inhibitors such as zosuquidar and tariquidar (13, 14) show these agents to be safer and more specific but with limited improvement in outcomes in the tumor types studied.

In the screening of novel compounds that might have an impact on steroid membrane trafficking, the substituted quinoline HG-829 was identified as a potent and selective inhibitor of Pgp-mediated drug resistance. Our studies show that HG-829 is a potent, noncompetitive inhibitor of drug export by Pgp that overcomes MDR in vitro and in vivo.

Materials and Methods

Study drug, reagents, and antibodies

HG-829 (PGEZ79901H) is a Pgp modulator synthesized by Procter and Gamble as a candidate inhibitor of androgen membrane trafficking. HG-829 is a substituted quinoline used as a hydrochloride salt to improve solubility (Fig. 1A). Tissue
culture medium was purchased from Invitrogen; FBS from Mediatech, Inc.; Matrigel from BD Biosciences; and rhodamine 123, cyclosporin A, daunorubicin, doxorubicin, vinblastine, paclitaxel, vincristine, etoposide, and G418 were purchased from Sigma-Aldrich. Calcein AM was obtained from Molecular Probes. Antibody against human Pgp, CD243-PE, and its respective negative control were purchased from Beckman Coulter. Pgp antibody for Western blotting was purchased from Santa Cruz Biotechnology and β-actin from Sigma-Aldrich.

Cell lines
K562 human chronic myeloid leukemia blast phase cells were obtained from the American Type Culture Collection. NCI-H460 cells were obtained from the National Cancer Institute (NCI) drug screening laboratories. Pgp-overexpressing, daunorubicin-resistant K562 cells (K562-R) were cultured with 60 ng/mL of daunorubicin once a week. Pgp-overexpressing, vinblastine-selected NCI-H460/VBL cells were cultured with 22.72 ng/mL vinblastine once weekly. Both K562 and H460 were cultured in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin. Experiments were carried out with cells cultured 7 to 10 days in drug-free media. Human embryonic kidney cells (HEK-293) stably transfected with empty pcDNA 3.1 vector (HEK-293pc) or vector containing ABCB1 (MDR-19 cells), ABCCI (MRP1 cells), and ABCG2 (BCRP cells) were maintained in minimum essential media containing 10% FBS, 1% penicillin/streptomycin, and 2 mg/mL G418 (15).

Resistant and susceptible cell lines were routinely confirmed by morphology, MTT, and Western blotting.

Pgp antibody staining
Pgp expression was detected by flow cytometry. Cells were washed 3 times in cold Staining Buffer (BD Biosciences) and stained with CD243-PE or negative control for 30 minutes at room temperature. Cells were washed with staining buffer and run on a FACScan flow cytometer (488-nm laser, 585/42BP; BD Biosciences). FlowJo 8.8.6 software was used to analyze the data (Tree Star, Inc.).

Functional assays
Cells were resuspended in complete media (phenol-red-free minimum essential media with 10% FBS), with 0.5 µg/mL rhodamine 123 with or without HG-829 and incubated at 37°C in 5% CO₂ for 30 minutes. After incubation, the cells were washed once in Dulbecco’s PBS (DPBS) and placed on ice in the dark or were resuspended in rhodamine-free complete media with or without HG-829 or cyclosporin-A and incubated at 37°C in 5% CO₂ for a 1-hour efflux period. In other investigations, cells were pretreated for 1 hour with the modulator, washed 2 times with DPBS, and incubated with 0.5 µg/mL rhodamine followed by incubation in rhodamine-free media for up to 8 hours. After the efflux period, the cells were washed with DPBS and placed on ice. A FACScan flow cytometer (Becton Dickinson) with a 488-nm argon laser was used to analyze sample fluorescence. Rhodamine 123
fluorescence was collected using a 530-nm bandpass filter. A minimum of 10,000 events was collected per sample. The samples were gated on forward scatter versus side scatter to exclude debris, and dead cells were excluded by propidium iodide staining (16). Each experiment was repeated at least 3 times.

Calcein AM experiments were carried out as previously described (17). Cells were washed 3 times with Krebs-HEPES buffer (1.5 mmol/L CaCl₂, 5.6 mmol/L glucose, 10 mmol/L HEPES, 4.7 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1.1 mmol/L MgSO₄, 118 mmol/L NaCl, pH 7.4) and then 90 μL plated into black 96-well plates and incubated at 37°C in 5% CO₂ for 30 minutes with 10 μL of different concentrations of the test compound. Calcein AM (0.3 μmol/L) was added to each well. Fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 520 nm on a Synergy HT (Bio-Tek Instruments) every 120 seconds. Competition assay analysis was conducted as previously described (18).

Fluorescence was detected in the same way, and on the basis of one-phase exponential curve fitting, the upper plateau (Y_max value) of each fluorescence–time curve was determined. Nonlinear regression using the 4-parameter logistic equation with variable Hill slope (GraphPad Prism version 5.01) was used to generate concentration–response curves. Data were normalized using the slopes from fluorescence–time curves, setting the lowest determined single value to 0% and the highest value as 100%. Each experiment was repeated at least 3 times.

**Immunoblotting**

Cells were harvested, washed in PBS (3 times), and lysed in 1× RIPA buffer containing 250 μmol/L NaOAc, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.2 μg/mL pepstatin A, and 500 μmol/L phenylmethylsulfonyl fluoride. One hundred micrograms of lysate was loaded on 7.5% PAGE and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes, blocked in 5% dry milk solution, and immunoblotted with indicated antibodies. Membranes were developed using ECL according to manufacturer’s protocols (Thermo Scientific).

**Proliferation assays**

In a 96-well plate, approximately 14 × 10⁵ K562-R, 4.5 × 10⁵ H460/VBL, 8 × 10⁵ transfected HEK-293 cells, and their respective parental cells were plated in 180 μL with or without modulators in complete media for at least 4 hours before different concentrations of the drug were added. HEK-293–transfected cells were allowed to adhere overnight before addition of the drug. After 72 hours of treatment, 50 μL of MTT substrate (2 mg/mL in PBS) was added and incubated for 4 hours at 37°C. Optical densities were read at 570 nm on a Synergy HT. Mean values and SEM were generated from 3 replicates at each drug concentration and reported as the percentage absorbance of control. The magnitude of sensitization to oncolytics by Pgp modulators was represented as the "sensitization factor," calculated as the quotient of the IC₅₀ for the oncolytic with vehicle divided by the corresponding IC₅₀ value in the presence of modulator.

**Murine xenograft studies**

Animals were maintained in accordance with the Institutional Animal Care and Use Committee of the University of South Florida (Tampa, FL) procedures and guidelines. Female severe-combined immunodeficient (SCID) beige mice (CB17. B6-Prkdcscid Lyst bg/Crl), 5 to 7 weeks old, were purchased from Charles River and allowed to acclimate in the animal facility for 1 week with a standard light–dark cycle and continuous access to food and water. K562 parental and K562-R cells resuspended in PBS and an equal volume of Matrigel were injected s.c. in the right flank (10 × 10⁶ in 100 μL). Tumor volumes were determined by measuring the length (l) and width (w) and calculating the volume (V = l × w² / 2). When tumors reached approximately 100 to 200 mm³, the animals were randomly assigned to treatment groups: (i) daunorubicin 2 mg/kg intraperitoneally (i.p.) every other day; (ii) daunorubicin 2 mg/kg i.p. + HG-829 25 mg/kg i.p.; (iii) HG-829 25 mg/kg i.p. daily; and (iv) HG-829 vehicle. Seven female mice were used for each experimental condition. HG-829 and HG-829 vehicle were administered continuously via osmotic pumps (model 2004, ALZET, Durect Corporation). The animals were treated for 12 days. Pumps were filled with HG-829 dissolved in a mixture of 35% Cremophor EL, 35% ethanol, 30% water or with solvents only. A 2-cm long vinyl catheter was attached to the pumps and kept in sterile 0.9% NaCl solution for 40 hours at 37°C before implantation, which was conducted 24 hours before initiation of daunorubicin treatment. Implantation of the pump was conducted under sterile conditions on animals anesthetized by inhalation with 3% isoflurane for induction followed by 2% during surgical manipulation. The osmotic pumps were placed s.c. on the dorsal surface near the shoulder blade, and the catheter was placed through a cutaneous entry created by a 16-gauge needle through the peritoneum into the peritoneal cavity. The skin was closed with sterile surgical sutures. Body weights were recorded every other day. Animals were observed daily for mortality and signs of ill health (weight loss, change in appetite, or behavioral changes).

**Statistical analysis**

ANOVA and Tukey multiple comparison tests were used to compare differences between groups in the proliferation assay, calcein AM functional assay, and tumor volume measurements. Differences between rhodamine intracellular values were compared by the Student t test. The IC₅₀ values were calculated using GraphPad Prism version 5.01 from Windows (GraphPad Software) by nonlinear regression analysis.

**Results**

**Pgp expression**

Increase in Pgp expression of resistant cells was detected by flow cytometry. Figure 1B shows a characteristic histogram of Pgp expression on K562-R cells (shaded dark histogram) compared with the parental cell line (shaded light histogram) that superposes to the negative control histogram and unstained cells. HG-829 has no significant effect on Pgp expression of the resistant and sensitive cells lines with 1 μmol/L HG-829 for 48 hours (Fig. 1B; K562-R, heavy solid
line; parental cell, dotted line). Although we observed a modest increase in Pgp detection by flow cytometry, there were no discernible changes in total Pgp by Western blotting (Fig. 1C) or by quantitative PCR (Supplementary Table S1).

**Functional assays**

We first examined the ability of HG-829 to inhibit Pgp-mediated rhodamine 123 transport. After a 1-hour incubation in rhodamine-free media, intracellular rhodamine concentration as measured by cellular fluorescence in the Pgp-overexpressing cell lines, K562-R and MDR-1–transfected HEK-293 cells (MDR-19; B) were incubated with 0.5 μg/mL rhodamine 123; cells were then washed and incubated in rhodamine-free medium with or without modulators for 1 hour. Both cell lines export rhodamine (shaded histogram) after rhodamine intake (dotted histogram). Rhodamine efflux is inhibited in the presence of HG-829, 2.5 μmol/L in the media (heavy solid line; K562-R, P = 0.03; MDR-19, P < 0.01) and to a lesser degree in the presence of 2.5 μmol/L cyclosporin A (CsA; dashed lines). Representative results from 1 of at least 3 experiments are shown. C, HG-829 significantly inhibited rhodamine efflux at concentrations as low as 0.07 μmol/L. Values represent mean ± SEM of 4 independent experiments. Efflux comparison versus media alone: HG-829 2.5 μmol/L = 0.002; 1.25 μmol/L, P = 0.003; 0.62 μmol/L, P = 0.002; 0.31 μmol/L, P = 0.002; 0.15 μmol/L, P = 0.01; 0.07 μmol/L, P = 0.02; cyclosporin A 2.5 μmol/L, P = 0.023. The zero values represent rhodamine intake (dotted) and efflux (white) without any modulator (D) HG-829 shows prolonged inhibition of Pgp-mediated rhodamine efflux. Cells were incubated with 2.5 μmol/L HG-829 or cyclosporin A for 1 hour, followed by 0.5 μg/mL rhodamine 123. Control samples were incubated with rhodamine alone. Cells were then washed and incubated in rhodamine-free medium. HG-829 promoted rhodamine retention for at least 8 hours (1 hour, P < 0.0001; 2 hours, P < 0.0001; 4 hours, P = 0.00374; 8 hours, P = 0.0145). Cyclosporin A only showed statistically significant retention at 1 hour (P = 0.0002). Values represent media ± SEM of 3 independent experiments (*, P < 0.05; **, P < 0.01; ***P < 0.001; ****P < 0.00001).

HG-829, K562-R cells were preincubated with HG-829 for 1 hour, followed by rhodamine for 30 minutes, then washed and resuspended in rhodamine and modulator-free media. Inhibition of rhodamine efflux was demonstrable up to 8 hours after resuspension (P < 0.05; Fig. 2D). When the cells were preincubated with cyclosporin A, we observed a rapid and progressive decrease in rhodamine retention with effective blockade of rhodamine export for only 1 hour (P < 0.001; Fig. 2D). When HG-829 was extensively washed before rhodamine intake, Pgp inhibition persisted for at least 4 hours in K562-R cells (P = 0.0001) and MDR–1–transfected HEK-293 cells (P < 0.001). Preincubation with both modulators, HG-829 and cyclosporin A, significantly increased rhodamine intracellular intake, in K562-R (P < 0.0001 and P = 0.002 for HG-829 and cyclosporin A, respectively) and MDR–1–transfected HEK-293 cells (P < 0.001). Preincubation with both modulators, HG-829 and cyclosporin A, significantly increased rhodamine intracellular concentration (Table 1); however, those preincubated with cyclosporin A showed 75% rhodamine efflux in K562–R cells (P = 0.002) and 95% in MDR cells (P = 0.0003). Both K562 parental and HEK-293 cells transfected with empty vector (i.e., Pgp-negative cells) accumulated rhodamine without change in...
retention in the presence or absence of modulators (data not shown).

Similarly, we tested Pgp activity with the fluorimetric measurement of the intracellular accumulation of calcein produced by ester hydrolysis of the Pgp substrate calcein AM. K562-R cells overexpressing Pgp showed characteristically low intracellular fluorescence levels over time (Fig. 3A, circles). Intracellular fluorescence significantly increased after HG-829 exposure in K562-R cells at all time points investigated ($P < 0.001$; Fig. 3A). Representative data at 19-minute incubation are shown in Fig. 3B. HG-829 treatment significantly increased cellular fluorescence compared with control (HG-829, 0.5 $\mu$mol/L, $P = 0.0015$; 1 $\mu$mol/L, $P = 0.0019$). Cyclosporin A significantly increased fluorescence accumulation at only 1 $\mu$mol/L ($P = 0.05$). These functional data suggest that HG-829 is not a Pgp substrate or competitive inhibitor of Pgp-mediated drug efflux.

To further discern the precise interaction of HG-829 with Pgp to inhibit drug efflux, we assessed concentration-dependent enzyme kinetics for calcein AM efflux through application of the Lineweaver–Burk linearization technique (17, 18). The $y$-intercept of the graph is equivalent to the inverse of the maximum reaction velocity ($V_{max}$), whereas the $x$-intercept of the graph represents $-1/K_{m}$ (Michaelis–Menten constant). Competitive inhibitors share the same $y$-intercept, whereas there are different slopes and $x$-intercepts between the 2 data sets. Noncompetitive inhibition-produced plots have the same $x$-intercept as the uninhibited enzyme, as $K_{m}$ is unaffected, but display disparate slopes and $y$-intercepts. The findings in Fig. 3C are consistent with the latter or noncompetitive P-glycoprotein inhibition by HG-829.

### Table 1. HG-829 inhibits Pgp-mediated rhodamine efflux in K562-R and MDR-19 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K562-R</th>
<th>MDR-19</th>
</tr>
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<tbody>
<tr>
<td>Unstained</td>
<td>2.05 ± 0.14</td>
<td>2.33 ± 0.16</td>
</tr>
<tr>
<td>Rhodamine intake</td>
<td>270.30 ± 14.33</td>
<td>367.00 ± 50.12</td>
</tr>
<tr>
<td>Efflux in media</td>
<td>14.10 ± 0.76</td>
<td>59.33 ± 4.16</td>
</tr>
<tr>
<td>Efflux with HG-829, 2.5 $\mu$mol/L</td>
<td>89.13 ± 4.47a</td>
<td>149.40 ± 9.30b</td>
</tr>
<tr>
<td>Efflux with cyclosporin A, 2.5 $\mu$mol/L</td>
<td>68.67 ± 4.46b</td>
<td>130.70 ± 15.65c</td>
</tr>
<tr>
<td>Rhodamine intake + HG</td>
<td>2,777.00 ± 49.52</td>
<td>1,839.00 ± 56.57</td>
</tr>
<tr>
<td>Efflux in media</td>
<td>2,538.00 ± 78.54</td>
<td>1,392.00 ± 113.50c</td>
</tr>
<tr>
<td>Rhodamine intake + cyclosporin A</td>
<td>1,556.00 ± 197.50</td>
<td>1,996.00 ± 129.10</td>
</tr>
<tr>
<td>Efflux in media</td>
<td>92.93 ± 4.42b</td>
<td>434.30 ± 12.86b</td>
</tr>
</tbody>
</table>

**NOTE:** Geometric mean fluorescent intensities of rhodamine efflux were analyzed; addition of HG-829 to efflux media significantly reduced rhodamine efflux in both cell lines (K562-R, $P < 0.0001$; MDR-19, $P = 0.0009$). Cyclosporin A displayed less effective reduction in rhodamine efflux (K562-R, $P = 0.0003$; MDR-19, $P = 0.0116$). Exposure to the Pgp modulators before rhodamine intake increased intracellular rhodamine fluorescence in both cell lines (K562-R, HG-829, $P < 0.0001$, cyclosporine A, $P = 0.0029$; MDR-19, HG-829, $P < 0.0001$, cyclosporin A, $P = 0.0033$). Preincubation with HG-829 completely inhibited rhodamine efflux in K562-R cells ($P = 0.0617$) and by 76% in MDR-19 cells ($P = 0.02$). Preincubation with cyclosporin A enhanced rhodamine retention by only 5% in K562-R cells ($P = 0.0018$) and less than 25% in MDR-19 cells ($P = 0.0003$).

$^aP < 0.0001$

$^bP < 0.001$

$^cP < 0.05$

HG-829 sensitizes $ABCB1$–overexpressing cells to antineoplastics

We investigated the sensitivity of $ABCB1$-overexpressing drug-resistant and -sensitive parental cells to HG-829 by changes in cytotoxicity upon exposure to antineoplastic substrates in a 72-hour MTT proliferation assay. Addition of HG-829 to antineoplastic substrates for Pgp enhanced drug-induced cytotoxicity in K562-R cells in a concentration-dependent fashion (Supplementary Fig. S1 and Supplementary Table S2). As shown in Figs. 4 and 5, $ABCB1$-expressing cells displayed 30- to 10,000-fold higher IC$_{50}$ values for the Pgp substrates daunorubicin, vinblastine, vincristine, paclitaxel, and etoposide than the corresponding parental cells (Tables 2 and 3). Coincubation with HG-829 at concentrations of 0.5 and 1 $\mu$mol/L completely reversed drug resistance in each of the cell lines tested. In K562-R cells, daunorubicin cytotoxicity was restored to a level approaching that of the parental line at an HG-829 concentration of 0.5 $\mu$mol/L (IC$_{50}$ = 10$^{-7}$ mol/L media, $1.76 \times 10^{-7}$ mol/L, sensitization factor, 57$	imes$; $P < 0.01$) and was minimally augmented at the higher 1 $\mu$mol/L concentration (IC$_{50}$ = 1.03 $\times 10^{-7}$ mol/L, sensitization factor, 97$	imes$; $P < 0.01$; Fig. 4A and Table 2, daunorubicin). Cyclosporin A at an equimolar concentration (1 $\mu$mol/L) was less effective (IC$_{50}$ = 3.65 $\times 10^{-7}$ mol/L, $P < 0.05$) with a sensitization factor of only 27$	imes$. Cyclosporin A at 0.5 $\mu$mol/L (IC$_{50}$ = 6.73 $\times 10^{-7}$ mol/L) did not show a statistically significant change in cytotoxicity (Table 2, daunorubicin). Resistance in the MDR-1–transfected HEK-293 cell line, MDR-19, was also completely inhibited by HG-829 (IC$_{50}$ = 1.04 $\times 10^{-6}$ mol/L media; 9.16 $\times 10^{-9}$ mol/L with HG-829, 0.5 $\mu$mol/L, $P < 0.01$; and
mo/\ell with HG-829, 0.5 \mu{\text{mol/\ell}}; 1.37 \times 10^{-11} \text{ mo/\ell} with HG-829, 1 \mu{\text{mol/\ell}}; 9.88 \times 10^{-12} \text{ mo/\ell} with cyclosporin A 0.5 \mu{\text{mol/\ell}}; and 4.99 \times 10^{-13} \text{ mo/\ell} with 1 \mu{\text{mol/\ell}} cyclosporin A, \( P < 0.001 \), confirming the substrate-dependent interaction of cyclosporin A. Potentiation of cytotoxicity by HG-829 extended to all other antineoplastics tested, including paclitaxel, vincristine, and etoposide (Fig. 5A–C and Table 3, paclitaxel, vincristine, and etoposide). In contrast, cyclosporin A did not enhance the cytotoxicity of paclitaxel and only slightly enhanced cytotoxicity of vincristine, and etoposide; however, this was not statistically significant (Table 3, paclitaxel, vincristine, and etoposide).

The activity of HG-829 against other non-Pgp ABC transporters, such as MRP1 (ABCC1) and breast cancer resistance protein (ABCG2), was evaluated in HEK-293–transfected cells. HG-829 displayed only moderate concentration-dependent sensitization of cells expressing either transporter at concentrations up to 2 and 4 \( \mu{\text{mol/\ell}} \), respectively (Fig. 5D and E and Table 3, vincristine and mitoxantrone).

### Efficacy in human xenografts

The ability of HG-829 to reverse Pgp-mediated MDR in vivo was evaluated in K562-R and parental cell xenografts implanted in female SCID beige mice treated with daunorubicin. Intraperitoneal treatment with daunorubicin alone had no significant effect on the growth rate of K562-R xenografts, whereas coadministration of HG-829 restored the antitumor activity of daunorubicin (\( P < 0.01 \); Fig. 6A). Daunorubicin significantly reduced tumor volumes in mice bearing the parental xenografts (\( P < 0.05 \) daunorubicin vs. HG-829 alone, \( P < 0.01 \) daunorubicin vs. HG-829 vehicle; Fig. 6B), and coadministration of HG-829 did not enhance the antitumor effects. Importantly, HG-829 was well tolerated either alone or in combination, as evidenced by the absence of differences in weight change compared with the vehicle or HG-829–treated cohorts. Of interest, animals treated with the combination of HG-829 and daunorubicin had less weight loss than those treated with daunorubicin alone. Animals bearing K562-R xenografts treated with daunorubicin, however, had a significant decrease in weight compared with the other 3 treatment groups (\( P < 0.001 \)). Similarly, in animals implanted with K562 parental xenografts treated with daunorubicin, weight loss was greater than that of the daunorubicin + HG-829 combination (\( P < 0.05 \)) and animals treated with HG-829 alone (\( P < 0.001 \), data not shown). Of interest, treatment with HG-829 alone modestly reduced tumor growth in animals bearing the sensitive (\( P = 0.09 \)) or resistant xenografts (\( P = 0.03 \)).

### Discussion

Enhancement of anticancer activity of conventional antineoplastics or restoration of chemosensitivity in resistant cancer cells is challenged by the redundancy of cellular defense mechanisms contributing to drug resistance. MDR encompasses a complex phenotype characterized by cellular resistance to a wide range of structurally unrelated, and mechanistically diverse, antineoplastics that can arise from the activation of genes involved in transmembrane drug...
transport, cell adhesion, intercellular communication, detoxification, and vesicle transport, as well as repression of genes involved in apoptosis (21). Studies with P-glycoprotein inhibitors are not only relevant for MDR; they could also be used to increase oral absorption of substrate compounds or to potentially increase brain penetration. A number of inhibitors of ABCB1-mediated MDR have been developed, and many have been tested in the clinical setting with limited success. Most of these compounds, such as cyclosporin A, act as competitive substrate inhibitors (22). Given that substrates interact with Pgp at multiple distinct sites (7), it is not surprising that capacity to suppress drug efflux by competitive inhibitors is substrate dependent. Cyclosporin A displayed remarkable drug-dependent variability. There was no enhancement of

![Graphs showing the effects of HG-829 on antineoplastic cytotoxicity](image)

**Figure 4.** HG-829 enhances antineoplastic cytotoxicity in cells that overexpress Pgp. K562-R (A); MDR-1–transfected HEK-293 cells (MDR-19; B); H460/VBL (C). Cells were treated with HG-829 or cyclosporin A as control and the respective drug for 72 hours, then incubated with MTT dye, and quantified for the level of MTT uptake. Parental cells (open circles); resistant cells (filled circles); HG-829 0.5 μmol/L (triangles); and HG-829 1 μmol/L (hatched squares). The mean of 4 wells was calculated for each concentration of the drug and reported as the percentage of control. Data represent media ± SEM of 2 independent experiments. Each experiment was repeated 4 to 6 times.

![Graphs showing the effects of HG-829 on antineoplastic cytotoxicity](image)

**Figure 5.** HG-829 enhancement of antineoplastic cytotoxicity extends to a wide range of Pgp substrates, including paclitaxel (A), vincristine (B), and etoposide (C) and cells overexpressing MRP1 (ABCG1; D) or breast cancer resistance protein (ABCG2; E). Cells were treated with HG-829 and corresponding antineoplastic for 72 hours, then incubated with MTT dye, and quantified for level of MTT uptake. Parental cells (open circles); resistant cells (filled circles); HG-829 0.5 μmol/L (triangles); HG-829 1 μmol/L (hatched squares); HG-829 2 μmol/L (inverted triangles); HG-829 3 μmol/L (open rhomboids); and HG-829 4 μmol/L (asterisks). The mean of 4 wells was calculated for each concentration of the drug and reported as the percentage of control. Data represent media ± SEM of 2 independent experiments. Each experiment was repeated 4 to 6 times.
Table 2. HG-829 enhances antineoplastic cytotoxicity in cells that overexpress Pgp

<table>
<thead>
<tr>
<th>Cells/modulator</th>
<th>IC50, mol/L</th>
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<tbody>
<tr>
<td>A. Daunorubicin</td>
<td></td>
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<tr>
<td>K562-R</td>
<td>1.00E-05</td>
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<tr>
<td>HG 0.5 μmol/L</td>
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<td>HG 1.0 μmol/L</td>
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<td>97.00</td>
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<td>CsA 0.5 μmol/L</td>
<td>6.73E-07</td>
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<td>27.45</td>
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<td>B. Doxorubicin</td>
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<tr>
<td>MDR-19</td>
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<td>HG 0.5 μmol/L</td>
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<td>113.48</td>
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<td>112.54</td>
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<tr>
<td>CsA 0.5 μmol/L</td>
<td>2.81E-07</td>
<td>3.70</td>
</tr>
<tr>
<td>CsA 1.0 μmol/L</td>
<td>2.01E-07b</td>
<td>5.17</td>
</tr>
<tr>
<td>HEK-293pc</td>
<td>7.71E-09</td>
<td></td>
</tr>
<tr>
<td>C. Vinblastine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H460-VBL</td>
<td>1.02E-08</td>
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<td>HG 0.5 μmol/L</td>
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<td>483.59</td>
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<td>CsA 1.0 μmol/L</td>
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<td>24,799.68</td>
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<tr>
<td>H460-S</td>
<td>4.72E-12</td>
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</table>

NOTE: (A) K562-R treated with daunorubicin. (B) MDR-1 transfected HEK-293 cells treated with doxorubicin (MDR-19). (C) H460/VBL treated with vinblastine. Cells were treated with HG-829 or cyclosporin A and the respective drug for 72 hours, then incubated with MTT dye, and quantified for the level of MTT uptake. Data represent mean ± SEM of 2 independent experiments. The relative SF was calculated as the quotient of the IC50 for the oncolytic with vehicle divided by the corresponding IC50 value in the presence of modulator.

Abbreviations: CsA, cyclosporin A; SF, sensitization factor.

aP < 0.01.
bP < 0.05.
cP < 0.001.

doxorubicin, paclitaxel, vinblastine, and etoposide cytotoxicity in resistant cell lines; however, vinblastine resistance was effectively reversed in H460/VBL cells, a result that was similar to data reported by de Souza and colleagues (23). Our investigations show that the novel substituted quinoline, HG-829, is a potent and selective inhibitor of ABCB1-mediated MDR, with comparable activity in reversing resistance in both drug-selected and ABCB1-transfected cells that extends to all Pgp substrates investigated, including daunorubicin, doxorubicin, paclitaxel, vinblastine, vincristine, and etoposide. HG-829 had weak inhibitory activity against other ABC transporters such as MRP1 (ABCC1) and breast cancer resistance protein (ABCG2), indicating that the modulatory effects of HG-829 are primarily Pgp-specific. Although treatment with HG-829 was associated with a modest increase in Pgp detected by flow cytometry using the UIC2 antibody, there was no change in total Pgp demonstrable by immunoblot analysis. Prior investigations have shown that substrate interaction with Pgp elicits conformational...
changes that unmask external epitopes recognized by UIC2 by flow cytometry (24, 25). The absence of discernible changes in total Pgp protein by immunoblot suggests that interaction of HG-829 with Pgp unmasks conformation-sensitive epitopes, thereby increasing accessibility for antibody recognition. Our findings that HG-829 completely reversed resistance to a wide range of structurally unrelated compounds in ABCB1 expressing cells and the ability to induce both rhodamine123 and calcein AM retention suggests that HG-829 inhibits drug export in a noncompetitive, substrate-independent manner. Our analysis of enzyme kinetics for calcein AM efflux supports noncompetitive inhibition (Fig. 3C); however, the precise mechanism of inhibition remains under investigation. Compared with cyclosporin A, HG-829 displayed greater potency at equimolar concentrations, showing a more than 10-fold enhancement of cytotoxicity in highly resistant cells. Enhancement of daunorubicin cytotoxicity in multidrug-resistant cells was also confirmed in vivo in murine xenografts. Coadministration of HG-829 restored antitumor activity of daunorubicin in K562-R xenografts compared with either daunorubicin (P < 0.01) or HG-829 alone. Surprisingly, treatment with HG-829 alone modestly reduced tumor growth in animals bearing the sensitive or resistant xenografts. Given that HG-829 had no discernible impact on tumor cell viability in vitro, these data suggest that the intrinsic antitumor effects of HG-829 in vivo relate to effects on tumor microenvironment or cells in the supportive niche, perhaps by modifying Pgp-mediated cytochrome P450 elaboration.

Consistent with the cytotoxicity data, drug accumulation studies showed that HG-829 significantly enhanced intracellular accumulation of daunorubicin in ABCB1-overexpressing cells while having no effect in parental cells (data not shown). Cellular efflux of rhodamine123 or calcein AM was completely suppressed at HG-829 concentrations as low as 0.07 μmol/L. Moreover, unlike the results with the competitive Pgp inhibitor cyclosporin A, suppression of both rhodamine 123 and calcein AM efflux after 1-hour exposure to HG-829 was sustained after washing and resuspending resistant cells in drug-free media for up to 8 hours. These findings indicate that the activity of HG-829 is not dependent upon continuous drug exposure (as opposed to competitive inhibitors) while promoting sustained suppression of Pgp transport capacity. These data suggest that HG-829 acts as a novel noncompetitive and sustained inhibitor of Pgp transport function, perhaps by promoting allosteric changes that preclude or impair drug binding and transport (26). Further investigation in clinical trials may be warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G. Caceres, S.M. Sebti, A.F. List
Development of methodology: G. Caceres, N.J. Lawrence
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Caceres
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Caceres, L. Sokol, K.L. McGraw, N.J. Lawrence, M. Wiebe
Writing, review, and/or revision of the manuscript: G. Caceres, B.W. Robey, L. Sokol, K.L. McGraw, N.J. Lawrence, A.F. List
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Clark
Study supervision: A.F. List

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