Gefitinib Modulates the Function of Multiple ATP-Binding Cassette Transporters *In vivo*

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Abstract

The 4-anilinoquinazoline (4-AQ) derivative gefitinib (Iressa) is an oral epidermal growth factor receptor tyrosine kinase inhibitor. Oral administration of 4-AQ molecules, such as gefitinib, inhibits ATP-binding cassette (ABC) transporter–mediated drug efflux and strongly increases the apparent bioavailability of coadministered drug molecules that are transporter substrates. Based on *in vitro* studies investigating 4-AQ interactions with several transporters, these effects have primarily been attributed to the inhibition of breast cancer resistance protein (BCRP; ABCG2). Although 4-AQ shows *in vitro* inhibition of P-glycoprotein [multidrug resistance protein (MDR1); ABCB1], the *in vivo* effect on this and other transporters is not known. In our studies, pretreatment of *Abcg2*$^{-/-}$ and *Mdr1(a/b)*$^{-/-}$ mice with gefitinib increased oral absorption and decreased systemic clearance of topotecan, a model substrate, indicating that additional transporters were inhibited. These results were extended to human orthologues using engineered cell lines to show that gefitinib inhibited the efflux of BCRP and MDR1 substrates and restored vincristine sensitivity in MDR1-expressing cells. Although gefitinib inhibited BCRP more potently than MDR1 (10-fold), the inhibition of both transporters occurred at clinically relevant concentrations (e.g., 1-5 μmol/L). These studies illustrate the broad implications for the therapeutic combination of gefitinib or other 4-AQ molecules with agents that are BCRP and MDR1 substrates. 4-AQ molecules may offer a means to increase the low and variable oral drug absorption of transporter substrates while decreasing interpatient variability and reversing tumor drug resistance. (Cancer Res 2006; 66(9): 4802-7)

Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are small molecules with potent cytostatic activity in select tumors (1). The 4-anilinoquinazoline (4-AQ) derivatives gefitinib (Iressa) and erlotinib (Tarceva) are tyrosine kinase inhibitors with Food and Drug Administration approval for the treatment of patients with locally advanced or metastatic non–small-cell lung cancer after failure of at least one prior chemotherapy regimen. These compounds have promise as single agents but strong rationale exists for combining them with standard anticancer agents. Tyrosine kinase inhibitors exert their action through competition with ATP for binding at the catalytic domain of tyrosine kinase enzymes such as EGFR. *In vitro* studies using biochemical and cell assays showed that tyrosine kinase inhibitors also interact with and modulate the function of the ATP-binding cassette (ABC) transporters breast cancer resistance protein (BCRP) and multidrug resistance protein (MDR1; refs. 2, 3).

Results of murine pharmacokinetic studies have shown that gefitinib increased the apparent bioavailability of the anticancer agent irinotecan, which is a BCRP substrate (4). As gefitinib affinity towards MDR1 is ~10-fold lower than that for BCRP (3), the pharmacokinetic effects of gefitinib have primarily been attributed to its interaction with BCRP alone (4, 5). However, MDR1 is highly expressed in the small intestine, liver, kidney, and brain, as well as in tumors (6–8). Therefore, it is important to understand the *in vivo* interaction between transport proteins and tyrosine kinase inhibitor molecules like gefitinib, and how this interaction may alter the systemic and cellular pharmacokinetics of substrate molecules. Here we used transporter-deficient mice and cell-based assays to show that a representative tyrosine kinase inhibitor compound, gefitinib, abrogates the function of both BCRP and MDR1 at clinically relevant dosages. These data will improve our understanding of the pharmacologic interactions between tyrosine kinase inhibitor compounds and ABC transporters, especially when these agents are prescribed in combination with other anticancer agents.

Materials and Methods

Drug formulation and administration. Topotecan (GlaxoSmithKline, King of Prussia, PA) was prepared in sterile water for injection (0.4 mg/mL). Gefitinib (AstraZeneca, Aldley Park, Cheshire, United Kingdom) was dissolved in DMSO (10% v/v final concentration) and diluted in carboxymethylcellulose (0.25% w/v) to 20 mg/mL (4).

Immunohistochemistry. Avidin-biotin-chromogen methods were used for BCRP detection (4). Human tissues were obtained from Ambion (Austin, TX) and ResGen/Invitrogen (Carlsbad, CA). Rhesus tissues were provided by Dr. Tim Mandrell (University of Tennessee Health Science Center, Memphis, TN). The monoclonal antibody BXP-53 (Kamiya Biomedical, Seattle, WA) was used to detect Bcrp1 in mouse brain (9). All other tissues were stained with a rabbit polyclonal antibody (rpABCG2) raised against a conserved mouse and human peptide sequence (10).

Pharmacokinetic studies in mice. All experiments were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Female CB17/Icr *scid*$^{-/-}$ [severe combined immunodeficient (SCID)] mice were from Charles River Breeding Laboratories (Wilmington, MA). *Abcg2*$^{+/+}$ and *Abcg2*$^{-/-}$ mice were on a C57Bl/6-129 background (11). *Mdr1(a/b)*$^{+/+}$ and *Mdr1(a/b)*$^{-/-}$ mice on an FVB background were purchased from Taconic (Germantown, NY). Topotecan disposition after a short infusion in the lateral tail vein or by oral gavage

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was evaluated as a single agent (2 mg/kg) or after a single oral gefitinib dosage (100 mg/kg). For the pharmacokinetic studies in SCID mice, blood (~1 mL) was collected from three animals per time point, as indicated in the figures. For the pharmacokinetic studies with transporter-deficient mice, blood (60-100 μL) was removed from the orbital plexus with a heparinized glass pipette twice during the experiment and via cardiac puncture at the terminal time point. All blood samples were handled and processed as described previously (12).

Pharmacokinetic analysis. Topotecan pharmacokinetic parameters were calculated using the NONMEM program (version V) as described previously (4). A two-compartment model was fit to the topotecan plasma lactone concentration versus time data, and parameters estimated included clearance (CL), volume (V), bioavailability (F), absorption rate constant (K_a), and intercompartmental rate constants (K_{cp}, K_{pc}). Nonlinear mixed-effect modeling was used to estimate the topotecan pharmacokinetic parameters and to determine whether treatment with gefitinib was a significant covariate on these parameters. With this study design, estimates of intersubject variability were indeterminable. The intrasubject variability was fixed at a relative error of 10% (related to assay error) and an absolute error of 0.25 ng/mL (related to assay level of detection), and the intersubject variability was fixed at 15%. We used the following covariate model to determine whether gefitinib significantly affected the clearance and bioavailability of topotecan:

$$\mathrm{CL} = \Theta_1 + \mathrm{GEF} \times \Theta_2 \text{ and } F = \Theta_3 + \mathrm{GEF} \times \Theta_4$$

where GEF (gefitinib) is 0 when gefitinib is not present and 1 when it is present. To determine if gefitinib was a statistically significant covariate, we evaluated if the addition of gefitinib significantly reduced the $-2 \times$ log likelihood [e.g., a decrease of 3.84 indicates a significant difference ($P < 0.05$) based on the χ^2 test]. We also tested whether the variables representing the gefitinib effects (Θ_2, Θ_4) were significantly different than zero (i.e., $\Theta_i > 1.96 \times \mathrm{SE}_{\Theta i}$, $i = 2, 4$), indicating a significance based on the two-tailed t test.

Dye and drug accumulation studies. LLC-PK1 derivative cell lines expressing human MDR1 (L-MDR1) were a kind gift from Dr. Alfred Schinkel (Division of Experimental Therapy, The Netherlands Cancer Institute, Amsterdam, the Netherlands; ref. 13). BCRP-expressing Saos-2 cells and Saos-2 pcDNA3.1 cells have previously been reported (10). Dyes were purchased from Molecular Probes (Eugene, OR). Cells were incubated with dye-containing medium at 37°C for 45 minutes. Gefitinib was added at the indicated concentrations 15 minutes before the dye or drug incubation. All flow cytometry assays with Hoechst 33342 (10 μmol/L)–treated and calcein-AM (1 μmol/L)–treated cells followed the methods of Wierdl et al. (10).

Cytotoxicity assay. LLC-PK1 or L-MDR1 cells (3×10^3 per well) were plated in 100 μL growth medium per well in 96-well plates and allowed to attach for 24 hours. Vincristine (Sigma, St. Louis, MO) and gefitinib were added in another 100 μL of growth medium and cells were incubated for 36 hours. The proportion of viable cells was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Cytotoxicity was expressed as IC_{50} values calculated from full dose-response curves using Sigma Plot (Systat, Point Richmond, CA).

Gefitinib cellular accumulation studies. BCRP-expressing Saos-2 cells and Saos-2 pcDNA3.1 cells were allowed to grow for 48 hours before use. Cells were incubated with gefitinib (1.1, 11.1, and 22.2 μmol/L) for 60 minutes. Samples were processed and analyzed for gefitinib concentration using a validated high-performance liquid chromatography tandem mass spectrometry (MS/MS) assay method for plasma, which was modified for analysis of gefitinib in cell lysates (14).

Results

Distribution of Bcrp1 in mouse tissues. Previous studies have determined Bcrp1 localization in FVB mouse tissues; however, in part our studies used C57BL/6-129 and SCID mice, and the distribution of Bcrp1 was unknown in these strains. We assessed Bcrp1 localization in these mice by immunohistochemistry and, for comparison, we used human tissues. We evaluated BCRP expression in formalin-fixed paraffin-embedded intestine, kidney, liver, and brain tissue sections using a polyclonal antibody (10) that cross-reacts with both murine Bcrp1 and human BCRP (Fig. 1). Antibody specificity was validated using tissues from *Abcg2*$^{-/-}$ mice or an irrelevant rabbit immunoglobulin G (not shown). Saos-2 cell lines transfected with pcDNA3.1 or pcDNA3.1-BCRP were used to optimize antibody dilution and as negative and positive controls for human tissue. In these mice, Bcrp1 is prominently expressed in the apical face of the intestinal epithelium (Fig. 1*A*). It is also localized apically in the brush border of proximal convoluted tubules in the kidney and at the bile canaliculi (Fig. 1*A*). Bcrp1 staining in brain sections was localized to the apical face of the microvascular endothelium, but with weaker intensity. Unlike mice, human kidney tissue did not stain for BCRP (not shown). Moreover, brain sections from humans and rhesus

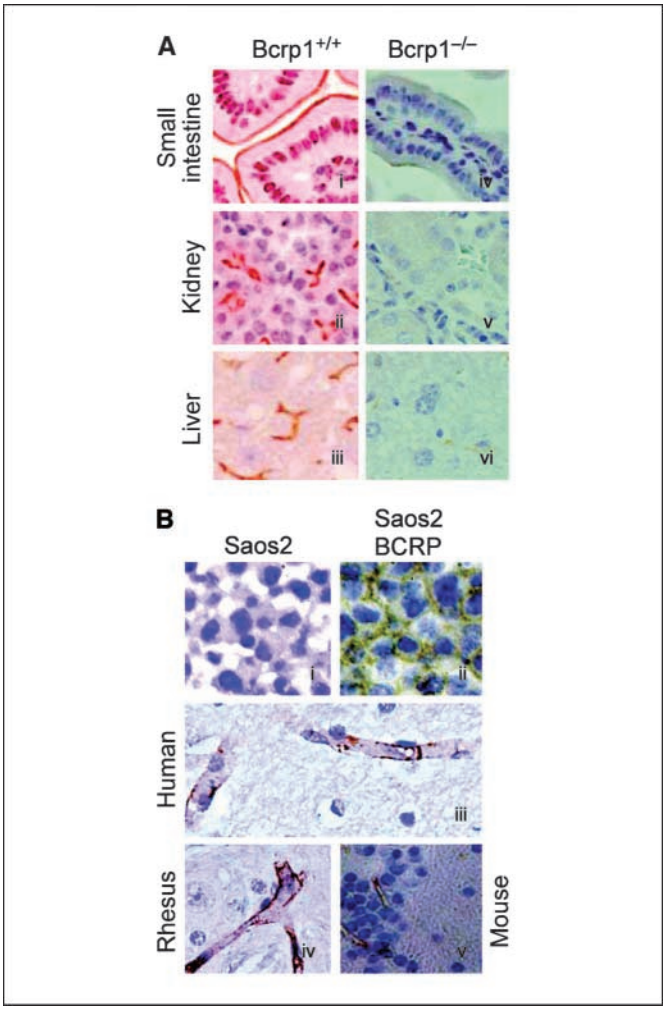

Figure 1. Bcrp1 is expressed in mouse and primate tissues. *A,* Bcrp1 expression in (SCID mouse) organs that influence drug absorption and elimination. Bcrp1 is expressed at the apical face of intestinal epithelium (*i*), at the apical face of proximal convoluted tubules in the kidney (*ii*), and the liver canaliculi (*iii*). Probing of sections from *Abcg2*$^{-/-}$ animals (*iv-vi*) with the primary antibody did not produce staining. *B,* BCRP expression in brain tissue. Antibody specificity in human tissue was tested using formalin-fixed paraffin-embedded Saos-2 pcDNA3.1 (*i*) and Saos-2 BCRP (*ii*) cell lines. Apical BCRP localization in brain endothelium was observed in human (*iii*), rhesus monkey (*iv*), and mouse tissues (*v*).

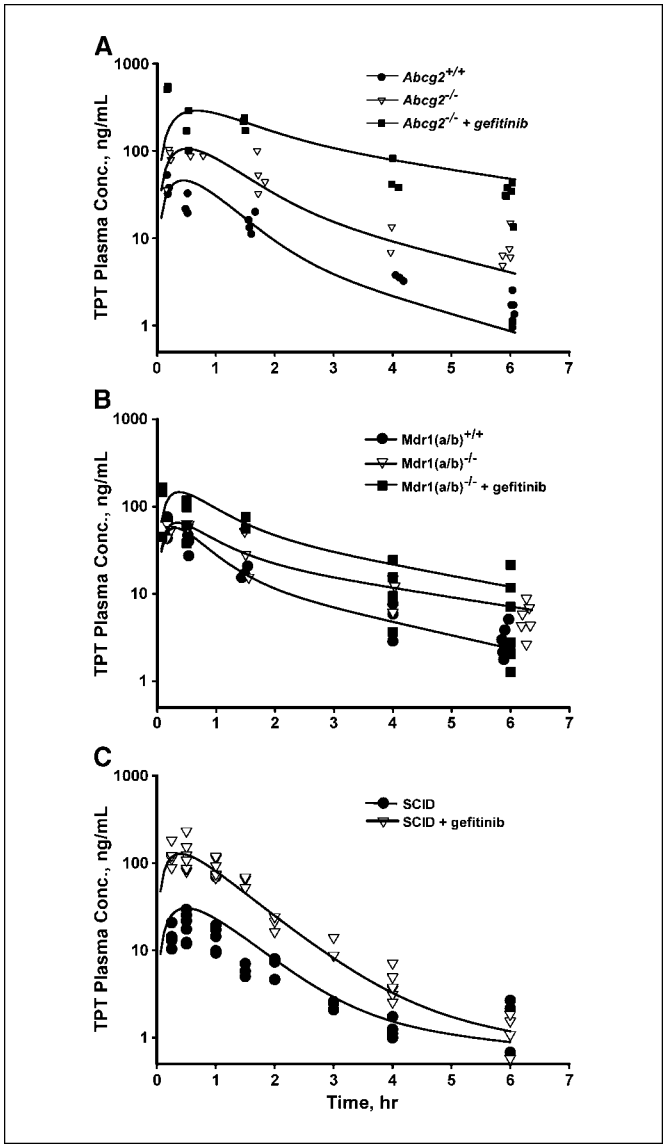

Figure 2. Gefitinib modulates the function of multiple ABC transporters leading to increased apparent bioavailability and reduced topotecan (*TPT*) clearance (see Table 1). An oral dose of topotecan (2 mg/kg) was administered alone or following a gefitinib oral dose (100 mg/kg) to *Abcg2*$^{+/+}$ and *Abcg2*$^{-/-}$ mice (*A*), *Mdr1(a/b)*$^{+/+}$ and *Mdr1(a/b)*$^{-/-}$ mice (*B*), and SCID mice (*C*).

monkey stained strongly in the apical face of the endothelium, which contrasts with the weaker expression in mouse brain (Fig. 1*B*).

Gefitinib modulates Bcrp1 function and other ABC transporters *in vivo*, leading to increased oral absorption and reduced topotecan clearance. We have previously shown that topotecan is orally absorbed and undergoes hepatic and renal elimination (12). *In vitro* and *in vivo* studies have shown that topotecan is transported by multiple ABC transporters (15, 16). In those studies, topotecan administration to *Abcg2*$^{-/-}$ and *Mdr1(a/b)*$^{-/-}$ mice has shown altered disposition. This suggests that topotecan is a good probe to assess interaction with these efflux transporters. To determine whether gefitinib was a specific inhibitor of Bcrp1 *in vivo*, we evaluated the pharmacokinetics of oral and i.v. topotecan (2 mg/kg) after a single oral gefitinib dose in *Abcg2*$^{-/-}$ mice (C57BL/6-129 background strain) and *Mdr1(a/b)*$^{-/-}$ mice (FVB background strain). Comprehensive pharmacokinetic analysis with nonlinear mixed-effect modeling was used to evaluate the data. The model was built considering genetic and pharmacologic ablation of transporters as covariates. Specifically, the effect of *Abcg2, Mdr1(a/b)*, or gefitinib was considered in a series of plasma topotecan pharmacokinetic experiments with wild-type and knockout animals that received topotecan (2 mg/kg) as an oral (see Fig. 2) or i.v. dose (not shown). As depicted in Fig. 2*A* and *B*, topotecan plasma concentrations were higher in *Abcg2*$^{-/-}$ and *Mdr1(a/b)*$^{-/-}$ mice, respectively, compared with strain-matched wild-type mice. Interestingly, topotecan plasma concentrations increased further in both knockout models when topotecan was administered immediately after a single oral dose of gefitinib (100 mg/kg). Detailed pharmacokinetic parameters (±SE) are listed in Table 1. Pharmacokinetic analysis showed that topotecan lactone systemic clearance in *Abcg2*$^{-/-}$ and *Mdr1(a/b)*$^{-/-}$ mice was lower than in wild-type mice and decreased further in knockout mice pretreated with gefitinib. Conversely, topotecan apparent bioavailability was higher in both knockout models and apparent bioavailability increased further with gefitinib treatment.

To assess the pharmacokinetic effect of gefitinib in wild-type mice, we administered topotecan to SCID mice, which we routinely use for drug efficacy studies. Topotecan disposition was evaluated after an oral or i.v. dose in SCID mice that had received a single oral gefitinib dose (100 mg/kg) or the gefitinib vehicle as a control. In these experiments, systemic topotecan clearance decreased by 46% in animals receiving a single oral gefitinib dose ($P < 0.0004$). Conversely, the apparent bioavailability increased by 74% ($P < 0.02$) in mice receiving gefitinib (Table 1).

Gefitinib modulates the function of human BCRP and MDR1. To evaluate if gefitinib modulated human MDR1 and BCRP function, we extended our studies with engineered cell lines (Fig. 3). We preincubated Saos-2 cell lines overexpressing BCRP with different gefitinib concentrations (0.1-25 μmol/L) and then with Hoechst dye. We also preincubated pig kidney epithelial cells (LLC-PK1) and LLC-PK1 cells engineered to overexpress MDR1 (L-MDR1) with gefitinib (0.1-25 μmol/L) and then with calcein-AM. The levels of intracellular fluorescence from Hoechst 33342 dye or calcein dye were detected using fluorescence-activated cell sorting (FACS) analysis of at least 10,000 cells. The accumulation of either dye was higher in the parent cell line than the transporter-expressing cell line. Moreover, dye accumulation was enhanced by gefitinib in a dose-dependent manner in the transporter-expressing cells (Fig. 3*A*). Representative FACS histograms and a quantitative estimation of the gefitinib effects are depicted in Fig. 3*B*.

The capacity of gefitinib to restore drug sensitivity was assessed with a cytotoxicity assay (MTT) using pig kidney derivative cell lines that stably express MDR1 (L-MDR1). As expected, parent cell lines (LLC-PK1) were sensitive to the MDR1 substrate vincristine (EC_{50} 0.12 μmol/L) whereas L-MDR1 cells were resistant (EC_{50} 3.8 μmol/L). Sensitivity to vincristine was restored to background levels (EC_{50} 0.10 μmol/L) when cells were co-incubated with vincristine and 10 μmol/L gefitinib (Fig. 3*C*). Gefitinib alone had no effect on cell growth as compared with cells growing in vehicle control (media and 0.1% DMSO) wells (not shown).

To assess if BCRP was transporting gefitinib at concentrations achieved in the clinical setting, we compared intracellular accumulation in Saos-2 pcDNA3.1 and Saos-2 pcDNA3.1-ABCG2 cells using a sensitive and specific high-performance liquid chromatography MS/MS method. Intracellular gefitinib concentrations

(Fig. 3*D*) were higher ($P < 0.05$) in the Saos-2 pcDNA3.1 cell line at the two lower concentrations (1.1 and 11.1 μmol/L) but we found no difference at 22.2 μmol/L. These results are in accord with published data in inverted membrane vesicles showing that gefitinib increases BCRP ATPase activity at submicromolar concentrations but is likely to act as an inhibitor at higher concentrations (3).

Discussion

Our study shows that administration of topotecan after a single dose of oral gefitinib increased topotecan apparent bioavailability and decreased systemic clearance in *Abcg2*$^{-/-}$ and *Mdr1(a/b)*$^{-/-}$ mice as compared with untreated knockout mice. This clearly indicates that gefitinib modulates intestinal transporters that efflux topotecan back into the intestine, thus allowing increased absorption. Furthermore, modulation of both renal and hepatic transporters is likely to contribute to the striking reduction in overall topotecan clearance in these mice. We extended our results, using engineered cell lines expressing human BCRP or MDR1, to show that gefitinib inhibited in a dose-dependent manner the function of each human transporter at clinically relevant doses (i.e., 1-5 μmol/L) even in cell lines engineered to express supraphysiologic levels of transporter. Two subtle points should be made. First, unbound gefitinib concentrations are not likely to reach those levels in plasma but will do so in the intestinal lumen following the oral dose. Second, the physiologic expression level in the intestinal epithelium will be much lower for each transporter. In addition, MDR1-mediated resistance to vincristine was abolished in these cells at clinically relevant gefitinib concentrations. This observation is in line with our previous results showing that gefitinib abolished BCRP-mediated resistance in cell lines and increased the antitumor efficacy of irinotecan in human tumor xenografts regardless of EGFR expression (4).

Consideration of the interaction of gefitinib with BCRP and MDR1 *in vivo* should be done in the context of the differential expression of these proteins in mice and humans. In accord with previous results (16), our immunohistochemistry studies in SCID mice showed that the distribution of Bcrp1 was high in intestine, liver, and kidney. However, BCRP is not expressed in the human kidney (17) and, as such, MDR1 inhibition may be more important in humans for the clearance of MDR1 substrates that undergo renal elimination. Furthermore, interaction of gefitinib with other renal and hepatic transporters (e.g., Mrp2-5) may also be important in the renal clearance of their drug substrates and should be investigated.

As subpopulations of patients expected to respond to tyrosine kinase inhibitor treatment are being identified, it is important to consider the effect that these compounds (i.e., gefitinib) might have on the transport of endogenous substrates. For example, the transport of androgens by BCRP has been reported (18). Recent clinical data showed that non–small-cell lung cancer patients receiving gefitinib had significantly lower plasma concentrations of testosterone and dehydroepiandrosterone. Furthermore, nonsmoking women, a subgroup that has a higher response rate to gefitinib (19), had significantly lower dehydroepiandrosterone-sulfate in their plasma as compared with their

Table 1. Summary of topotecan pharmacokinetic parameters in wild-type and transporter-deficient mice treated with topotecan alone or with topotecan plus gefitinib

Parameter	Treatment			*P*	
	Topotecan	Topotecan	Topotecan + gefitinib		
	Abcg2$^{+/+}$	*Abcg2*$^{-/-}$	*Abcg2*$^{-/-}$	(+/+) vs (−/−)	Gefitinib vs without
CL (L/h/m^2)	15.4 (1.6)	11.3 (2.2)	8.3 (2.5)	0.006	0.008
V_c (L/m^2)	5.75 (0.8)	—	—	—	—
F	0.11 (0.02)	0.22 (0.06)	0.47 (0.11)	0.06	0.006
K_{cp} (h^{-1})	0.50 (0.12)	—	—	—	—
K_{pc} (h^{-1})	0.62 (0.09)	—	—	—	—
K_a (h^{-1})	2.35 (0.40)	—	—	—	—
	Mdr1(a/b)$^{+/+}$	*Mdr1(a/b)*$^{-/-}$	*Mdr1(a/b)*$^{-/-}$	(+/+) vs (−/−)	Gefitinib vs without
CL (L/h/m^2)	15.5 (0.97)	12.2 (1.5)	10.2 (1.4)	0.004	0.04
V_c (L/m^2)	4.9 (0.47)	—	—	—	—
F	0.21 (0.02)	0.30 (0.04)	0.50 (0.10)	0.01	0.03
K_{cp} (h^{-1})	0.32 (0.04)	1.45 (0.41)	0.29 (0.57)	0.006	0.004
K_{pc} (h^{-1})	0.68 (0.06)	—	—	—	—
K_a (h^{-1})	2.02 (0.48)	—	—	—	—
		SCID	SCID	Gefitinib vs without	
CL (L/h/m^2)		42.2 (4.6)	22.5 (7.2)	0.0004	
V_c (L/m^2)		19.2 (3.7)	—	—	
F		0.35 (0.09)	0.61 (0.14)	0.02	
K_{cp} (h^{-1})		0.59 (0.19)	—	—	
K_{pc} (h^{-1})		0.18 (0.07)	—	—	
K_a (h^{-1})		1.43 (0.42)	4.41 (0.86)	0.00008	

NOTE: Data are presented as mean (SE). —, no significant difference.

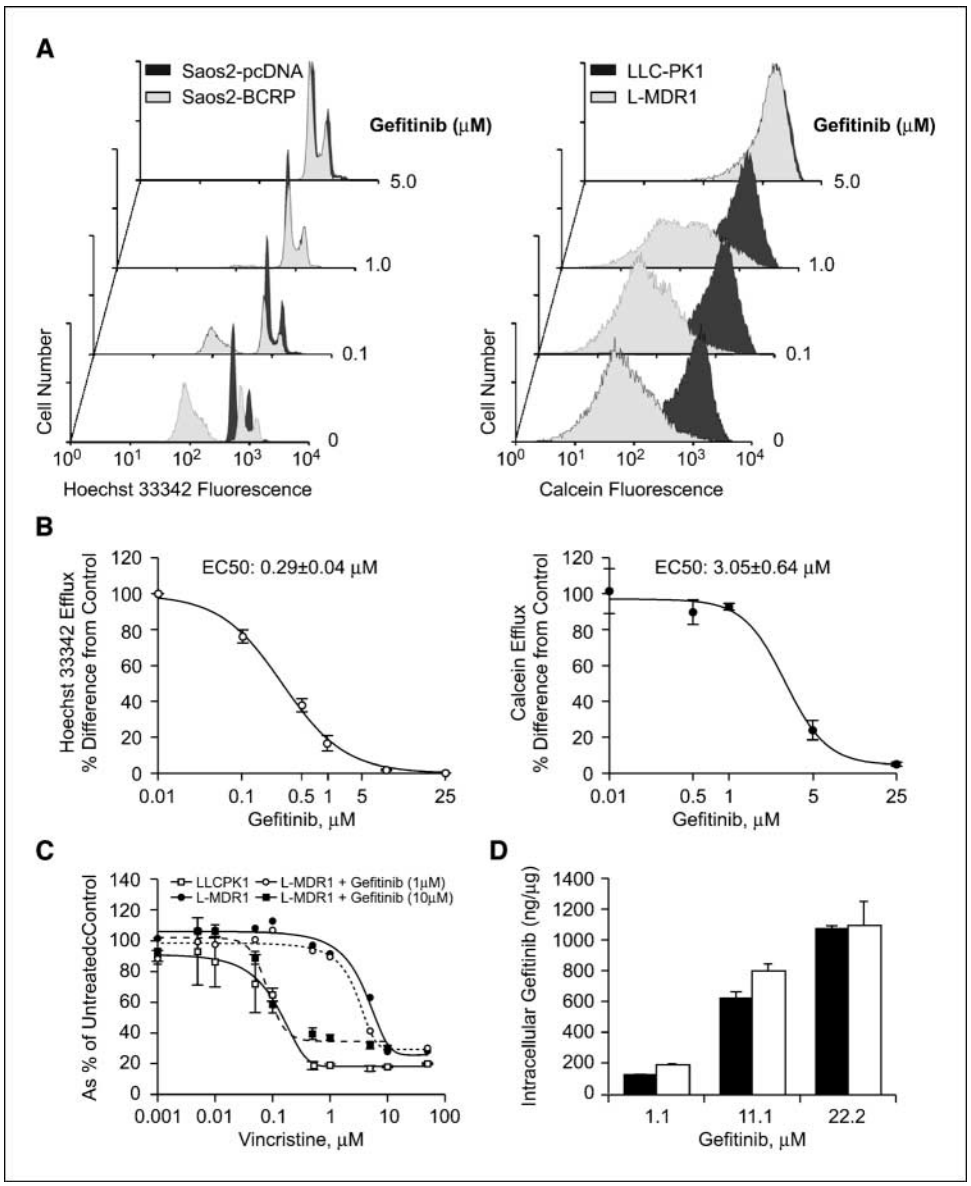

Figure 3. Gefitinib modulates the function of human BCRP and MDR1. *A,* accumulation of Hoechst 33342 dye in cells expressing human BCRP increased following incubation with gefitinib. Similarly, co-incubation with gefitinib increased accumulation of calcein in cells expressing MDR1. *B,* quantitative assessment of gefitinib effects from representative flow cytometry experiments. *C,* gefitinib abolished MDR1 resistance to vincristine. *D,* low intracellular accumulation in BCRP-expressing cells suggests gefitinib is a substrate at the low micromolar range but overall intracellular differences are minimal.

pretreatment values (20). It is possible that these differences observed after gefitinib treatment were due to inhibitory effects on transporters expressed in humoral tissues secreting androgens. Thus, gefitinib-associated decreases in serum androgen levels may be mediated by BCRP inhibition. Further investigation is needed to determine if this decrease plays a significant role in the clinical efficacy of gefitinib and if the efficacy is due to off-target effects.

Taken together, our results show that tyrosine kinase inhibitors, such as gefitinib, have the capacity to modulate multiple transporters *in vitro* and, more importantly, *in vivo*. As a consequence, clinical use of these agents should be evaluated accordingly when used in combination with cytotoxic regimens that tend to have narrow efficacy-toxicity windows.

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