Identification of Inhibitors of ABCG2 by a Bioluminescence Imaging–Based High-Throughput Assay

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Abstract

ABCG2 is a member of the ATP-binding cassette (ABC) family of transporters, the overexpression of which is associated with tumor resistance to a variety of chemotherapeutic agents. Accordingly, combining ABCG2 inhibitor(s) with chemotherapy has the potential to improve treatment outcome. To search for clinically useful ABCG2 inhibitors, a bioluminescence imaging (BLI)–based assay was developed to allow high-throughput compound screening. This assay exploits our finding that D-luciferin, the substrate of firefly luciferase (fLuc), is a specific substrate of ABCG2, and ABCG2 inhibitors block the export of D-luciferin and enhance bioluminescence signal by increasing intracellular D-luciferin concentrations. HEK293 cells, engineered to express ABCG2 and fLuc, were used to screen the Hopkins Drug Library that includes drugs approved by the Food and Drug Administration (FDA) as well as drug candidates that have entered phase II clinical trials. Forty-seven compounds showed BLI enhancement, a measure of anti-ABCG2 activity, of ≥5-fold, the majority of which were not previously known as ABCG2 inhibitors. The assay was validated by its identification of known ABCG2 inhibitors and by confirming previously unknown ABCG2 inhibitors using established *in vitro* assays (e.g., mitoxantrone resensitization and BODIPY-prazosin assays). Glafenine, a potent new inhibitor, also inhibited ABCG2 activity *in vivo*. The BLI-based assay is an efficient method to identify new inhibitors of ABCG2. As they were derived from a FDA-approved compound library, many of the inhibitors uncovered in this study are ready for clinical testing. [Cancer Res 2009;69(14):5867–75]

Introduction

ABCG2 is a recently described member of the ATP-binding cassette (ABC) transporters, a family of proteins that use the energy of ATP hydrolysis to transport certain chemicals out of cells (1, 2). The overexpression of ABC transporters has been associated with multidrug resistance (MDR), a major impediment to successful cancer chemotherapy. ABCG2 confers resistance to several chemotherapeutic agents, such as mitoxantrone, daunorubicin, doxorubicin, bisantrene, topotecan, and flavopiridol (3). In addition, ABCG2 has been found to affect drug transport across the gastrointestinal epithelium and the blood-brain barrier (4). Many believe that judiciously combining ABCG2 inhibitor(s) with standard cancer chemotherapy will nullify the protection tumor cells receive, preventing cancer survival and metastasis (2, 4, 5). However, that idea remains to be tested, largely due to the lack of suitable ABCG2 inhibitors, despite significant efforts at discovering them.

We recently showed that D-luciferin, the substrate of firefly luciferase (fLuc), is a specific substrate for ABCG2 (6). fLuc is the most commonly used reporter for imaging transgene expression *in vivo*. fLuc catalyzes the oxidation of D-luciferin, releasing photons that can be quantified through bioluminescence imaging (BLI; ref. 7). In cells that express both ABCG2 and fLuc, ABCG2 inhibitors, such as fumitremorgin C (FTC; ref. 8), block the export of D-luciferin and increase the effective intracellular concentration of D-luciferin that is accessible to fLuc, causing enhanced light output. BLI is an increasingly widespread imaging technique, provides an extremely high signal-to-background (S/B) ratio, and is easy to perform (9).

This study describes the development of a BLI-based high-throughput assay to screen for ABCG2 inhibitors. This assay was applied to screen a clinically relevant compound library established at Johns Hopkins University, namely, the Hopkins Drug Library (HDL; refs. 10, 11). We identified 47 compounds causing a greater than 5-fold increase in bioluminescence, consistent with marked ABCG2 inhibition. Some are known ABCG2 inhibitors. Many of those not previously known were confirmed using established methods.

Materials and Methods

Reagents. D-luciferin sodium salt was obtained from Gold Biotechnology, Inc. Verapamil, colchicine, and mitoxantrone were purchased from Sigma Chemical Co. BODIPY-prazosin was obtained from Invitrogen. Glafenine, flavoxate hydrochloride, and doxazosin mesylate were obtained from Sigma Chemical. FTC was a kind gift of Dr. S. Bates (National Cancer Institute). All compounds were prepared in DMSO for *in vitro* experiments. For *in vivo* experiments, ABCG2 inhibitor was dissolved in ethanol/cremophor EL/saline (1:1:6).

Cell lines. The establishment of ABCG2-overexpressing HEK293 cells stably transfected with CMV-luc2CP/Hygro (referred to here as HEK293/ABCG2/fLuc) has been described previously (12). Control empty vector–transfected HEK293 cells were stably transfected with CMV-luc2CP/Hygro in the same way and are referred to here as HEK293/empty/fLuc. Cells were cultured in MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. HEK293 cells stably transfected with the ABCG2-expressing construct were maintained in medium containing 1 mg/mL G418 and 100 μg/mL hygromycin B. ABCG2-overexpressing NCI-H460 human non–small cell lung carcinoma cells (National Cancer Institute, Frederick, MD) were established and characterized as described previously (13). They were maintained in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. All cultures were maintained at 37°C in a humidified 5% CO_2/95% air incubator.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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BLI assay. HEK293/ABCG2/fLuc cells were plated into 96-well plates at a density of 4 × 10^4/100 μL per well and allowed to attach overnight. The following day, 10 μL of each compound or the control solvent was transferred from a compound library in a 96-well, high-throughput format into the wells using a multichannel pipette. The final concentration of each compound was ~17 μmol/L. D-luciferin (5 μL; 1.2 mg/mL in PBS) was then added to achieve a final concentration of ~50 μg/mL. The plates were gently tapped to assure that all solutions were well mixed, and imaging commenced immediately. Images were taken every 5 min for ~1 h. Light output from each well was quantified at the 40-min time point after initiation of imaging, and the S/B ratio of the light output from each compound divided by that from the control well was calculated. This S/B ratio serves as an indicator of the potency of ABCG2 inhibition, the mechanism by which BLI signal is enhanced.

Table 1. Compounds with ≥5-fold BLI enhancement

Compound	Fold	Known therapeutic effect
A. Previously known ABCG2 inhibitors		
Gefitinib (39)	19.0	Antineoplastic
Harmine (40)	8.9	n/a
Prazosin (41)	8.4	Antihypertensive
Dipyridamole (18)	7.1	Antithrombotic
Curcumin (42)	6.3	Nutrient
Nelfinavir mesylate (37)	6.1	Antiviral
Niguldipine (43)	5.9	Antihypertensive
Riboflavin (44)	5.4	Antispasmodic
Reserpine (4)	5.4	Antihypertensive
Hesperetin (45)	5.0	Antispasmodic
B. Previously unknown as ABCG2 inhibitors		
Glafenine	20.6	Analgesic
Tracazolate	20	Sedative
Calcimycin (A23187)	16.9	Calcium ionophore
Doxazosin mesylate salt	15.9	Antihypertensive
Verteporfin	11.3	Ophthalmic
Flavoxate hydrochloride	11.2	Antispasmodic
Brij 30	11.1	n/a
Quinacrine	10.6	Anthelmintic
Grapefruit oil	10.6	n/a
Dihydroergotamine mesylate	9.6	Vasoconstrictor, specific in migraine
Harmaline	8.8	Central nervous system stimulant, antiparkinsonian
Clebopride maleate	7.9	Antiemetic, antispasmodic
Silver nitrate	7.7	Antibacterial
Isorhamnetin	7.0	n/a
Gramicidin A	6.9	Antibacterial
Clebopride	6.7	Antiemetic
Rotenone	6.7	Acaricide, ectoparasiticide, inhibits NADH2 oxidation to NAD
Clomiphene citrate	6.7	Gonad stimulating principle
Aromatic cascara fluid extract	6.6	Therapeutic plant
Sildenafil	6.6	Impotency therapy
Emodin	6.4	Antimicrobial, anticancer, cathartic
Flubendazole	6.3	Anthelminthic
Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone)	6.3	Diagnostic aid
Periciazine (propericiazine)	6.3	Antipsychotic
Isoreserpine	6.2	Antihypertensive
Acepromazine	6.2	Sedative
Flutamide	6.1	Antineoplastic
Podophyllum resin	6.1	Dermatologic
Gambogic acid	6.0	Antibacterial, inhibits HeLa cell growth *in vitro*
Piperacetazine	5.8	Antipsychotic
Digitoxin	5.7	Cardiotonic, cardiotoxic; inhibits Na^+/K^+ ATPase
Acetophenazine maleate	5.6	Antipsychotic
Eupatorin	5.6	Emetic ex *Eupatorium spp* and other *Compositae*
Estrone hemisuccinate	5.4	Estrogen
Raloxifene hydrochloride	5.4	Bone resorption
o-Dianisidine	5.0	Not approved
Oligomycin	5.0	Antibiotic, antifungal

Abbreviation: n/a, no therapeutic effect available.

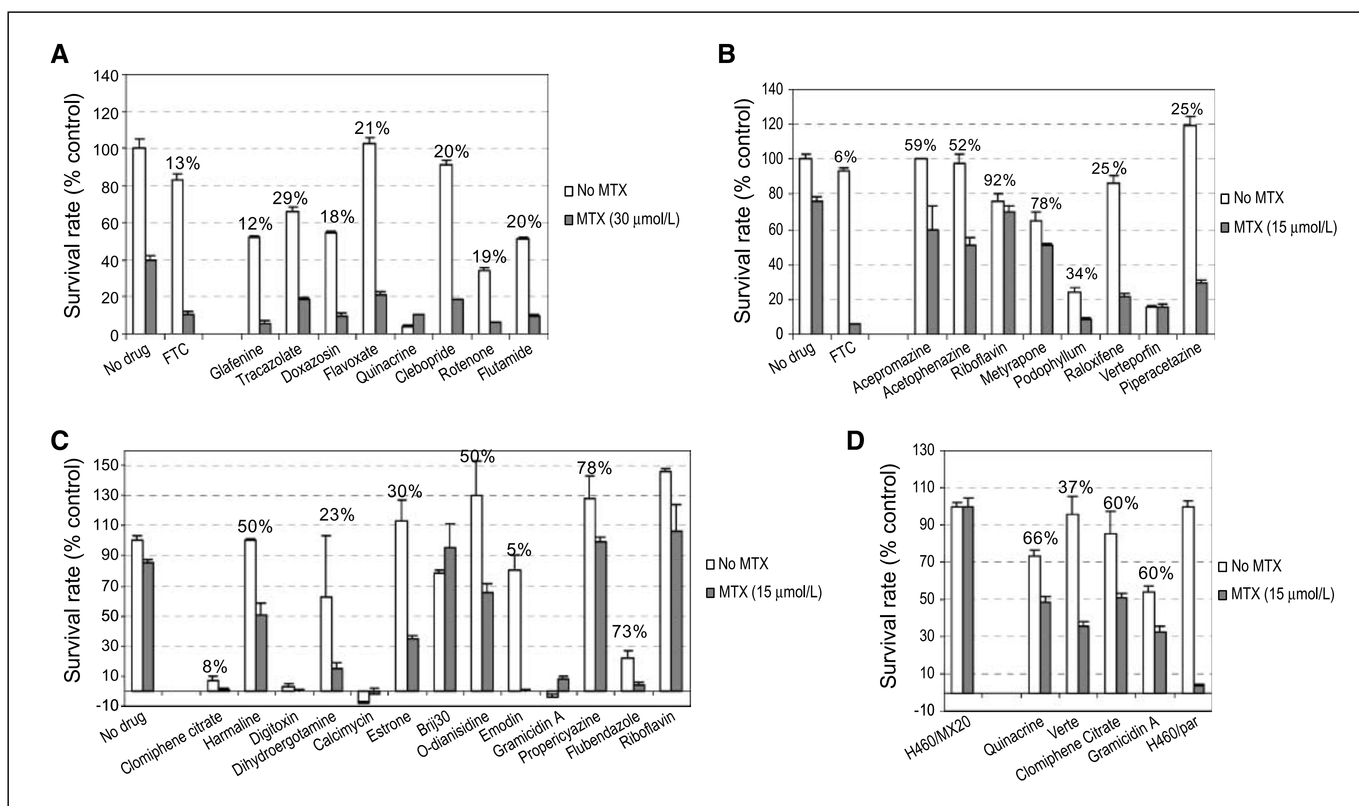

Figure 1. Mitoxantrone (*MTX*) resensitization assay. NCI-H460/MX20 (ABCG2-overexpressing, human non–small cell lung carcinoma cells) cells were treated for 3 d with or without mitoxantrone (30 μmol/L in *A* and 15 μmol/L in *B–D*) in the presence of a potential inhibitor (20 μmol/L in *A–C* and 1 μmol/L in *D*), and surviving cells were assessed with the XTT assay. Survival rates were expressed as percentages normalized by the data obtained in the negative control where no mitoxantrone or any compound was added. FTC (10 μmol/L) was used as a positive control. Numbers on top of bar pairs are survival rates caused by each compound in the presence of mitoxantrone relative to the compound alone. *Columns,* mean (n = 3); *bars,* SE.

Assay performance. Signal-to-noise ratio, S/B, and Z′ values, which indicate the robustness of the assay, were calculated as described previously (14). Background was defined as the light output from cells incubated with D-luciferin and the solvent only.

Resensitization assay. The ABC transporter–inhibiting ability of the potential inhibitors identified was further tested by evaluating their ability to resensitize ABCG2-overexpressing, human non–small cell lung carcinoma (NCI-H460/MX20) cells to mitoxantrone, or MDCKII cells overexpressing P-glycoprotein (Pgp) or MRP1, to colchicine. Cells were plated in 96-well plates at 1×10^4 per well and allowed to attach. Mitoxantrone was added to 15 or 30 μmol/L, with or without a putative ABCG2 inhibitor. Colchicine was added at 1 μmol/L for MDCKII/Pgp cells and 0.3 μmol/L for MDCKII/MRP1 cells. After 2 d of incubation, cell viability was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay as described previously (12). All results were normalized to a percentage of absorbance obtained in controls.

BODIPY-prazosin uptake assay. HEK293/ABCG2 cells were plated in six-well plates at a density of 1.1×10^6 per well and allowed to attach. Cells were then changed into medium containing 0.25 μmol/L BODIPY-prazosin (15), and compound to be tested was added to a final concentration of 20 μmol/L followed by incubation at 37°C for 1 h. Cells were then harvested, washed with ice-cold PBS once, resuspended in cold PBS, and analyzed with flow cytometry. Analyses were performed with FACScan (Becton Dickinson) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Ten thousand events were counted per sample. The resultant histograms were analyzed with CellQuest software (Becton Dickinson).

***In vivo* BLI.** Animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Both HEK293/ABCG2/fLuc and HEK293/empty/ABCG2 cells were implanted s.c. into 6-wk-old female nude mice at 1×10^6 at each site. The IVIS 200 small animal imaging system (Xenogen Corp.) was used for BLI and 2.5% isoflurane was used for anesthesia. D-luciferin was injected i.p. into mice at 150 mg/kg, and imaging was performed every few minutes for >1 h. ABCG2 inhibitor was administered via tail vein injection as a bolus during imaging, with imaging continued thereafter.

Data analysis. Living Image (Xenogen) and IGOR (Wavemetrics) image analysis software were used to superimpose and analyze the corresponding grayscale photographs and false color BLI images. Light intensities of regions of interest were expressed as total flux (photons/second). The IC_{50} values of identified ABCG2 inhibitors were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software) using variable-slope logistic nonlinear regression analysis. Data are presented as mean ± SE (n = 3).

Results

Statistical evaluation of the BLI-based assay. A HEK293/ABCG2/fLuc stable clone was chosen for this BLI-based high-throughput screen because FTC, a known specific inhibitor of ABCG2, enhances its BLI signal output significantly (8). The screen was performed in a 96-well format. HEK293/ABCG2/fLuc cells were plated from 1 to 8×10^4 per well and treated with solvent only or with FTC. D-luciferin concentrations varied from 20 to 100 μg/mL, and imaging data were acquired every 5 minutes for 1 hour. The quality of the BLI-based high-throughput screen assay was evaluated statistically as described previously (14). Z′ values were

calculated for each combination of parameters. An ideal assay is expected to produce $Z' = 1$, with $1 > Z' \geq 0.5$ reflecting a robust assay. The Z' values obtained from this assay ranged from 0.5 to 0.9.

Screening of the HDL using the BLI assay. The HDL is composed primarily of drugs approved by the Food and Drug Administration (FDA) and is the most complete library of clinically approved drugs (10, 11).

Images were taken every 5 minutes for ~1 hour, and light output from each well at the 40-minute time point was chosen for quantification. The S/B of the light output from each compound divided by that from the control well was calculated. This ratio was used as an indicator of the potency of ABCG2 inhibition, the mechanism by which BLI signal is enhanced.

The result of the full screen is presented in Supplementary Table S1. Two hundred and nineteen candidate ABCG2 inhibitors were identified from 3,273 compounds screened. Candidate inhibitors are defined as compounds producing at least 2-fold signal enhancement over control values. About 150 weaker inhibitors were also identified. Among the 219 potent (>2-fold signal enhancement) inhibitors, 88 (~40%) had not been previously reported as an inhibitor or substrate of any ABC transporter. The majority (~84%) of the ~150 weak inhibitors had not been previously reported as either inhibitors or substrates of ABC transporters. Forty-seven compounds showed signal enhancement of ≥5-fold (Table 1). Of those, 10 are known ABCG2 inhibitors or substrates (Table 1A), validating the assay. The identification of many previously reported ABCG2 inhibitors, including both potent and weak ones, such as gefitinib (16), reserpine (17), dipyridamole (18), and curcumin (19), suggests that the assay is sensitive. The most potent of the novel inhibitors, glafenine, enhanced the BLI signal by ~20-fold (Table 1B).

Confirmation of candidate ABCG2 inhibitors using established assays. We used two established assays, the mitoxantrone resensitization assay (1) and the BODIPY-prazosin fluorescent dye uptake assay (15), to confirm that compounds identified in the BLI screen were indeed ABCG2 inhibitors.

Twenty-eight novel candidate ABCG2 inhibitors identified in the BLI screen were tested by mitoxantrone resensitization assay, a hallmark of ABCG2 inhibitor function (4). Both ABCG2-overexpressing H460/MX20 cells and the parent line were treated with mitoxantrone (15 or 30 μmol/L) for 3 days. As expected, H460/MX20 cells survived exposure to mitoxantrone better than the parent cells due to the induced expression of ABCG2 (~40% versus ~9% survival in 30 μmol/L mitoxantrone and ~80% versus <20% in 15 μmol/L mitoxantrone). The potent, selective ABCG2 inhibitor FTC restored the sensitivity of H460/MX20 cells to mitoxantrone and significantly reduced their survival rate. Twenty-eight novel candidate inhibitors were initially tested at 20 μmol/L for 3 days. Twenty showed a similar capacity to sensitize H460/MX20 cells to mitoxantrone, confirming that they are indeed ABCG2 inhibitors (Fig. 1*A–C*). Brij 30 was found to resensitize H460/MX20 cells to mitoxantrone significantly after 2 days of incubation (data not

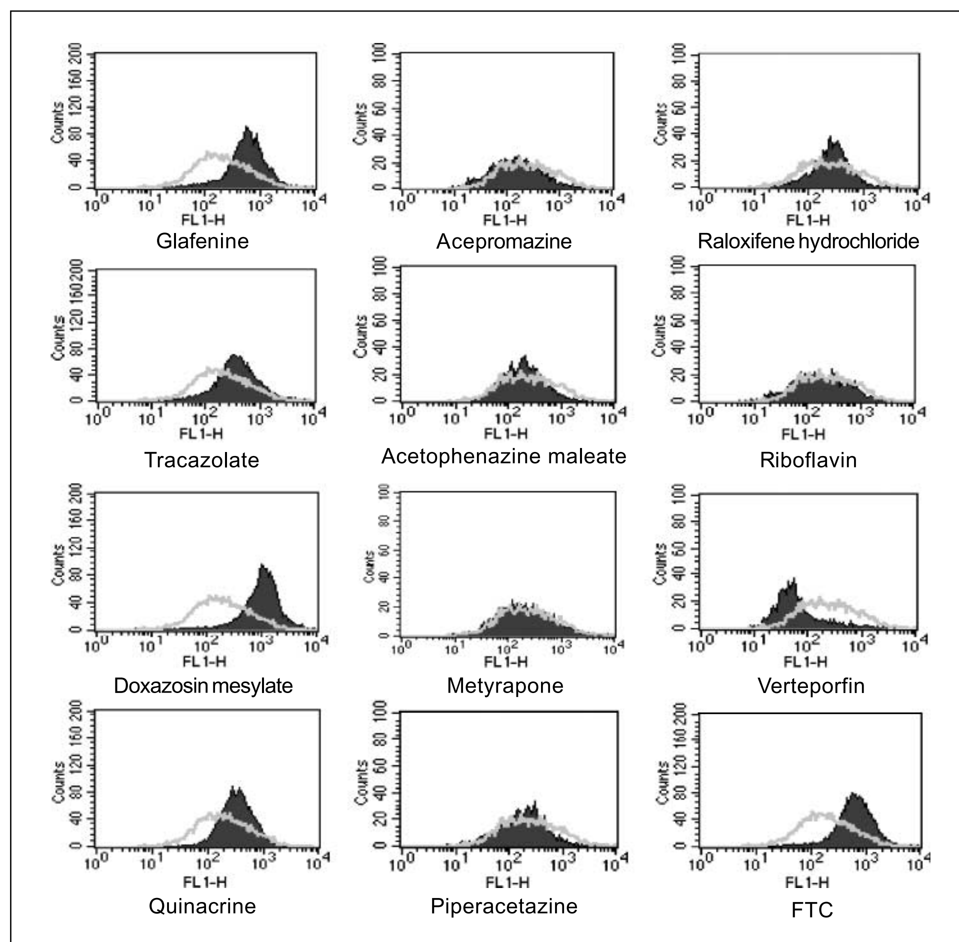

Figure 2. Effect of selected positive hits on ABCG2 function shown by flow cytometry analysis of the BODIPY-prazosin dye uptake assay. HEK293/ABCG2 cells were incubated in BODIPY-prazosin in the absence (*open curve*) or presence of a compound (20 μmol/L; *filled curve*) as described in Materials and Methods. FTC (10 μmol/L) was used as a positive control.

Table 2. Results of the mitoxantrone resensitization assay and the BODIPY-prazosin dye uptake assays

Compound	Mitoxantrone	BODIPY-prazosin
Glafenine	Yes	Yes
Tracazolate	Yes	Yes
Doxazosin mesylate	Yes	Yes
Verteporfin	Yes	No
Flavoxate hydrochloride	Yes	Yes
Quinacrine	Yes	Yes
Clebopride maleate	Yes	Yes
Metyrapone	Yes	No
Rotenone	Yes	Yes
Acepromazine	Yes	No
Flutamide	Yes	Yes
Podophyllum resin	Yes	Yes
Piperacetazine	Yes	No
Acetophenazine maleate	Yes	No
Raloxifene hydrochloride	Yes	No
Riboflavin	Yes	No

shown). The most active inhibitors were glafenine and doxazosin mesylate, which, at concentrations of 20 μmol/L, reduced the survival of H460/MX20 cells to 12% and 18%, respectively. These results were consistent with their considerable activity in the BLI screen (20- and 16-fold signal enhancement, respectively). That suggests that the magnitude of BLI signal enhancement can reflect the potency of ABCG2 inhibitors.

Six compounds, including quinacrine, verteporfin, digitoxin, clomiphene citrate, calcimycin, and gramicidin A, were too cytotoxic to be tested at 20 μmol/L (Fig. 1*A–C*). Each was tested again at 1 μmol/L with 15 μmol/L mitoxantrone for 2 days. At this lower concentration, quinacrine, verteporfin, clomiphene citrate, and gramicidin A showed resensitization of H460/MX20 cells to mitoxantrone, confirming them as ABCG2 inhibitors (Fig. 1*D*). The other two, digitoxin and calcimycin, were tested at even lower concentrations (0.3, 0.1, and 0.03 μmol/L). They were no longer cytotoxic at 0.1 and 0.03 μmol/L but did not reduce the survival rate of H460/MX20 cells significantly after 2 days when coincubated with 15 μmol/L mitoxantrone (data not shown). However, at these lower concentrations (0.03 and 0.1 μmol/L), they enhanced BLI signal minimally (data not shown).

In summary, 26 of the 28 candidate compounds identified in the BLI assay were confirmed by the mitoxantrone resensitization assay to be new ABCG2 inhibitors. Compounds designated as false positive were actually difficult to test in the mitoxantrone resensitization assay by virtue of their direct cytotoxicity and had to be tested at very low (noncytotoxic) doses below which their ability to inhibit ABCG2 was also compromised.

Sixteen of the candidate ABCG2 inhibitors were also tested with the BODIPY-prazosin assay. HEK293/ABCG2 cells were incubated with BODIPY-prazosin and each test compound and then subjected to flow cytometry. Nine of the 16 compounds, glafenine, tracazolate, doxazosin mesylate, quinacrine, clebopride, flutamide, flavoxate hydrochloride, rotenone, and podophyllum resin, were positive by this assay. Notably, seven compounds identified by the BLI assay and confirmed by mitoxantrone resensitization assay, acepromazine, acetophenazine maleate, metyrapone, piperacetazine, raloxifene hydrochloride, riboflavin, and verteporfin, were negative according to this assay (Fig. 2). However, all seven were confirmed by the mitoxantrone resensitization assay. The results of the mitoxantrone resensitization and the BODIPY-prazosin assays are compared in Table 2.

Sensitivity of the BLI assay. The BLI assay was further evaluated by searching the HDL for previously known ABCG2 inhibitors. Due to the relatively recent characterization of ABCG2, relatively few ABCG2 inhibitors are known (20). Twenty-five previously known ABCG2 inhibitors/substrates were found to be included in the HDL. In addition to the 10 compounds listed in Table 1A producing significant BLI signal, 15 additional known ABCG2 inhibitors are present in the HDL (Supplementary Table S2). Twenty-two of those compounds enhanced the BLI signal significantly (from 2.3- to 19-fold), and only 3, naringenin, acacetin, and genistein, enhanced the BLI signal <2-fold (1.9-, 1.8-, and 1.2-fold, respectively).

Further characterization of selected new ABCG2 inhibitors. As described previously (12), an ABCG2 inhibitor can enhance fLuc-based BLI signal in a dose-dependent manner. The BLI signal-enhancing effect of selected ABCG2 inhibitors was evaluated within the range of 0.001 to 100 μmol/L with HEK293/ABCG2/fLuc cells and 50 μg/mL D-luciferin. The data obtained at 40 minutes after imaging commencement were chosen arbitrarily to be plotted (Fig. 3*A*). The IC_{50} value of glafenine as an ABCG2 inhibitor was calculated to be 3.2 μmol/L. For three other ABCG2 inhibitors, doxazosin mesylate, flavoxate hydrochloride, and clebopride maleate, the BLI signal did not reach a plateau even at concentrations as high as 100 μmol/L. Assuming that the BLI signal produced by each compound at 100 μmol/L approaches a maximum value, the IC_{50} values of doxazosin mesylate, flavoxate hydrochloride, and clebopride maleate can be calculated to be 8.0, 20, and 8.2 μmol/L, respectively. The same assay was used to calculate the IC_{50} value of FTC, and it was determined to be 6.6 μmol/L using the 30-minute data. Although that value deviates from the IC_{50} values reported for FTC in literature (0.3–1.3 μmol/L), the discrepancy may be caused by the fact that the assays involve different substrates. In terms of its ability to inhibit ATPase, Robey and colleagues (13) measured the IC_{50} value of FTC to be 1 μmol/L,

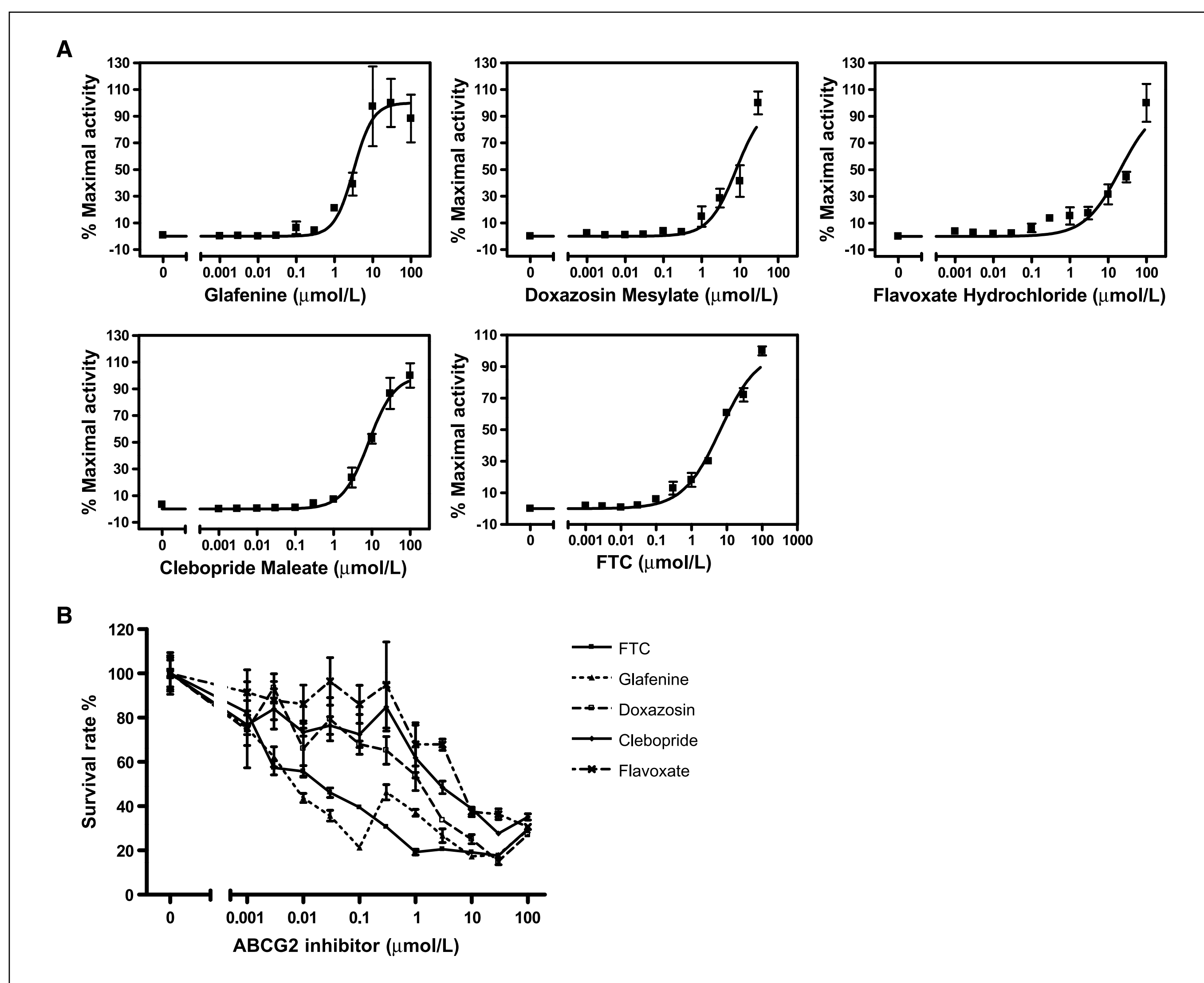

Figure 3. ABCG2 inhibitors cause a dose-dependent response in assays measuring the activity of ABCG2 inhibitors. *A,* enhancement of bioluminescence signal in HEK293/ABCG2 cells expressing fLuc. Cells were imaged in medium containing 50 μg/mL D-luciferin and increasing concentrations of glafenine, doxazosin mesylate, flavoxate hydrochloride, clebopride maleate, or FTC, and bioluminescence signal was quantified. The data were plotted and the IC_{50} value of each ABCG2 inhibitor was calculated with GraphPad Prism version 4.0 for Windows using variable-slope logistic nonlinear regression analysis. *B,* resensitization of ABCG2-overexpressing HEK293 cells to mitoxantrone treatment. Cells were plated at a density of 1×10^4 per well in 96-well plates and allowed to attach before incubation in medium containing an ABCG2 inhibitor and/or mitoxantrone for 3 d. Cell viabilities were assessed with the XTT assay and expressed as percentages of the control that was treated with mitoxantrone alone. *Points,* mean (n = 3); *bars,* SE.

whereas Özvegy and colleagues obtained values of 1.3 μmol/L (21) and 0.4 μmol/L (22). The IC_{50} value of FTC was also reported to be 0.8 μmol/L using the pheophorbide A fluorescent dye uptake assay (23). According to those previous reports, FTC reached the plateau of its ABCG2-inhibiting effect at a concentration of 10 μmol/L, but the BLI assay indicates that higher concentrations would be needed to provide a maximal inhibitory effect (Fig. 3*A*).

The dose-dependent effect of ABCG2 inhibitors was also evaluated with the mitoxantrone resensitization assay. ABCG2-overexpressing H460/MX20 cells were incubated for 3 days with increasing concentrations of each ABCG2 inhibitor in addition to 15 μmol/L mitoxantrone, and the survival rates were plotted against the concentration of each compound (Fig. 3*B*). Consistent with the IC_{50} values of each ABCG2 inhibitor obtained from BLI assay, glafenine proved a more potent ABCG2 inhibitor than FTC, doxazosin mesylate, clebopride maleate, and flavoxate hydrochloride.

To check whether newly identified ABCG2 inhibitors were specific to ABCG2 as opposed to inhibiting other MDR pumps generally, they were also tested for their ability to inhibit ABCB1/Pgp and ABCC1/MRP1. The resensitization assay was performed with MDCKII cells overexpressing Pgp or MRP1 (24) using colchicine, a Pgp and MRP1 substrate (25, 26). MDCKII cells overexpressing Pgp or MRP1 were incubated for 2 days in medium containing 1 μmol/L (for Pgp) or 0.3 μmol/L (for MRP1) of colchicine and increasing concentrations of each ABCG2 inhibitor. As shown in Supplementary Fig. S1A, compared with verapamil, glafenine is a more potent Pgp inhibitor, doxazosin mesylate has similar potency, and clebopride maleate and flavoxate hydrochloride show weak Pgp-inhibiting ability at relatively high concentration

(30 μmol/L). Glafenine and doxazosin mesylate have similar potencies to verapamil for MRP1 inhibition, whereas clebopride maleate and flavoxate hydrochloride proved weak even at relatively high concentration (30 μmol/L; Supplementary Fig. S1B). However, all of these ABCG2 inhibitors are specific for ABCG2 at low concentrations (≤1 μmol/L). For example, glafenine can effectively resensitize H460/MX20 cells to mitoxantrone at a concentration as low as 0.001 μmol/L (Fig. 1*B*) but does not provide resensitization of MDCKII/Pgp or MDCKII/MRP1 cells to colchicine until 1 or 10 μmol/L, respectively.

***In vivo* inhibition of ABCG2 activity by selected new ABCG2 inhibitors.** Two of the newly identified ABCG2 inhibitors, glafenine and doxazosin mesylate, were tested further for their ability to inhibit ABCG2 function *in vivo*. We have previously shown that administration of FTC *in vivo* can significantly enhance D-luciferin–dependent BLI signal output of xenografts derived from ABCG2-overexpressing HEK293 cells (6). Here, we used the same strategy to test the effect of these new ABCG2 inhibitors *in vivo*. HEK293/empty/fLuc and HEK293/ABCG2/fLuc cells were implanted s.c. into opposite flanks of female nude mice. Five mice were implanted to generate 10 ABCG2-overexpressing xenografts and 5 controls. Animals were imaged after D-luciferin administration, which was followed by a bolus injection of a single dose of ABCG2 inhibitor and continued imaging. After glafenine injection (25 mg/kg, i.v.), 9 of 10 ABCG2-overexpressing xenografts showed enhanced BLI signal over the control in the same mouse. Those 10 xenografts showed an average of ~2.9-fold signal enhancement over the control with the highest approaching 6.7-fold (Fig. 4). Glafenine caused increases in BLI signal of up to ~11.6-fold and ~17.4-fold in two separate HEK293/ABCG2/fLuc xenografts (right front and rear flanks) in the same mouse compared with the signals generated by those xenografts immediately before injection. By contrast, the BLI signal of the HEK293/empty/fLuc xenograft in the left flank increased by only ~2.6-fold (Fig. 4). Doxazosin mesylate injection caused a similar but weaker BLI signal enhancement of ABCG2-overexpressing xenografts *in vivo* (data not shown).

Discussion

We have developed and evaluated a cell-based, high-throughput assay to uncover new inhibitors of ABCG2. This assay builds on our finding that D-luciferin, the substrate of fLuc, is a specific substrate of ABCG2 and uses BLI to screen for ABCG2 inhibitors (6). The screening of 3,273 compounds identified 219 candidate ABCG2 inhibitors with at least a 2-fold signal enhancement over controls, ~60% of which have been previously reported as ABCG2 inhibitors, including gefitinib, prazosin, and harmine. The ability to identify known ABC transporter inhibitors, both potent and weak, proved that the assay is sensitive and reliable. The results also show the ability of the assay to identify previously unknown ABCG2 inhibitors. Approximately 40% of the 219 potent and ~84% of the ~150 less potent compounds have never been reported previously as being inhibitors or substrates of an ABC transporter. The less potent compounds, in particular, may be difficult to identify with other methods.

The power of the BLI assay was further shown by the confirmation of the majority (26 of 28) of the new ABCG2 inhibitors uncovered, indicating a low false-positive rate. Twenty-eight candidate ABCG2 inhibitors with over 5-fold signal enhancement were subjected to the mitoxantrone resensitization assay, and 16 of them were also tested with the BODIPY-prazosin dye uptake assay. All but two were confirmed by the mitoxantrone resensitization assay. The low false-positive rate may be explained by a previous report, where a screen of >70,000 compounds by an assay using pure fLuc found no activator of the luciferase-coupled reaction that could enhance the luminescent signal (27). The signal enhancement seen in our BLI assay is attributed only to the increased intracellular concentration of D-luciferin on administration of putative ABCG2 inhibitors from our library. However, caution is still required because ABCG2 may not be the only membrane transporter that affects the permeability of D-luciferin. In addition, pore-forming agents or detergents that disrupt cell membranes may cause false positives because of the leakage of D-luciferin into cells. Among the two compounds identified in this screen that could not be confirmed by the mitoxantrone resensitization assay, calcimycin is a cation ionophore and falls into this category.

The BLI-based assay is very sensitive with no false negatives obtained. Whereas the results of the mitoxantrone resensitization assay were as expected, those of the BODIPY-prazosin dye uptake assay were intriguing. Only 9 of 16 compounds tested were confirmed by this fluorescence-based assay and 7 (~44%) failed this assay altogether. All of the seven compounds were confirmed by the mitoxantrone resensitization assay (Table 2). Notably, mitoxantrone resistance is the hallmark of the ABCG2-inhibiting phenotype (28, 29); therefore, this discrepancy suggests a high

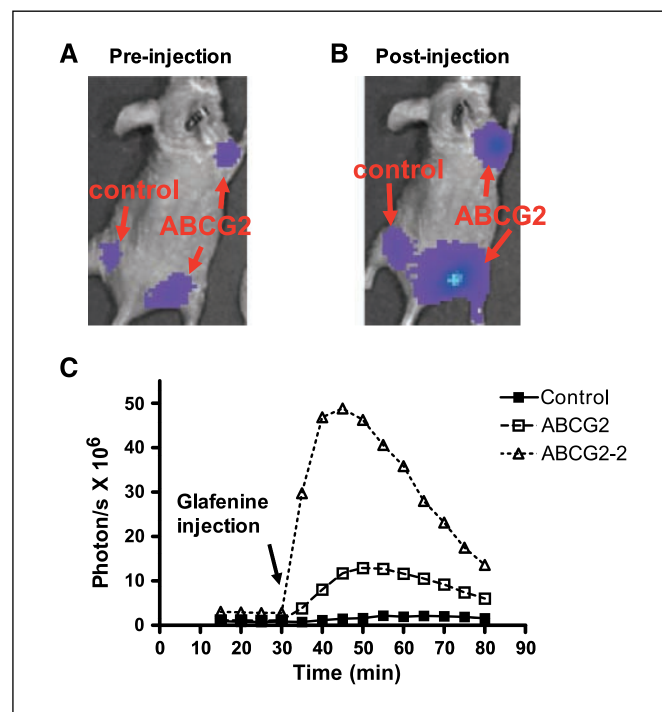

Figure 4. Glafenine inhibits ABCG2 activity in a living mouse as shown by BLI. HEK293/empty/fLuc (control) and HEK293/ABCG2/fLuc cells were implanted to the flanks (left and right, respectively) of immunocompromised (nude) mice. *A,* a representative mouse showing BLI acquired 30 min after administration of D-luciferin i.p. immediately before administration of glafenine (25 mg/kg). *B,* the same mouse as in *A* imaged 15 min after i.v. glafenine administration. *C,* time course of BLI signal from both control and ABCG2-overexpressing xenografts before and after glafenine injection. The BLI signal from ABCG2-transfected xenografts increased up to ~11.6-fold and ~17.4-fold (right front and rear, respectively), whereas the BLI signal from the control xenograft increased only ~2.6-fold compared with their signals immediately before glafenine injection. *Arrow,* time of glafenine injection.

false-negative rate for the BODIPY-prazosin assay. To understand the discrepancy better, we analyzed the structures of the seven compounds that could not be confirmed by the BODIPY-prazosin assay. Metyrapone, acepromazine, piperacetazine, and acetophenazine have an aromatic ketone functional group, which can act as an electron acceptor and deactivate the singlet state of BODIPY via an intermolecular electron transfer process (30, 31). Porphyrin in verteporin and benzopteridine in riboflavin can quench the fluorescence of BODIPY by way of photoinduced electron transfer (32, 33). It has been reported previously that the fluorescence of several dyes used to probe mitochondrial transmembrane potential can be quenched by some anticancer drugs, including adaphostin, mitoxantrone, and amsacrine (34). Accordingly, fluorescence-based assays must be cautiously applied. The implication of this finding is significant. Because fluorescence-based assays have seen the most use in discovering new ABCG2 inhibitors (13, 23, 35, 36), it is possible that many ABCG2 inhibitors that quench fluorescence have been missed. The BLI-based screening assay described here has the advantage of not being prone to such an artifact. That advantage was shown by our search of the HDL for previously known ABCG2 inhibitors, which revealed that the BLI-based assay missed none of them.

The BLI-based assay is efficient due to the elimination of incubation and wash steps. Several hundred drugs can be screened in 1 day using the current system, with many thousands of drugs possible if the technique were automated. False negatives caused by cytotoxicity in extended incubation are not a concern.

Candidate ABCG2 inhibitors obtained from our screen of the HDL are categorized based on their therapeutic effects and can be clustered into several classes, including drugs affecting the cardiovascular and central nervous systems, the gastrointestinal system, among others (Supplementary Table S3). Previously, it has been reported that ABCG2 is expressed in brain, colon, small and large intestine, venous endothelium, and capillaries, suggesting that the expression pattern indicates that ABCG2 plays a protective role in these tissues. However, further evidence is needed to support that hypothesis (4). Our discovery that many drugs treating diseases associated with these tissues are ABCG2 inhibitors is consistent with the possible functions of ABCG2 indicated by its expression pattern. The involvement of ABCG2 in some of the processes represented by the classes of agents uncovered in our assay has been recognized previously. Many anti-HIV drugs inhibit ABCG2 (37), and a bidirectional interaction between epidermal growth factor receptor and ABCG2 has been reported (38). The roles that ABCG2 may play in most of the other processes have not been studied extensively. Information obtained from this screen may shed light on new mechanisms of action of drugs designed for other purposes. Because most of the compounds in the HDL library have been approved by the FDA, certain candidates could be immediately used in conjunction with standard cancer chemotherapy to help combat the effects of ABC transporter expression, which often confound such treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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