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Advances in Brief

Pheophorbide a Is a Specific Probe for ABCG2 Function and Inhibition

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Abstract

Pheophorbide a (PhA), a chlorophyll catabolite, was shown to be an ABCG2 substrate based on Abcg2$^{-/-}$ knockout mouse studies (J. W. Jonker *et al.,* Proc. Natl. Acad. Sci. USA, *99:* 15649–15654, 2002). We developed a functional assay for ABCG2 using PhA and the ABCG2 inhibitor fumitremorgin C. In selected cell lines expressing high levels of P-glycoprotein, multidrug resistance-associated protein 1, or ABCG2, PhA transport was observed only in cells expressing ABCG2. Fumitremorgin C-inhibitable PhA transport was found to correlate with cell surface ABCG2 expression as measured by the anti-ABCG2 antibody 5D3. We found that 100 μM of the cyclin-dependent kinase inhibitor UCN-01 or 1 μM of the P-glycoprotein inhibitor tariquidar inhibited ABCG2-mediated PhA transport. In 4-day cytotoxicity assays, ABCG2-mediated resistance to SN-38 and topotecan was abrogated in ABCG2-transfected HEK-293 cells treated with 1 μM tariquidar, and ABCG2-transfected cells were 6–7-fold resistant to UCN-01. PhA is an ABCG2-specific substrate with potential value in measuring ABCG2 function and expression in clinical samples.

Introduction

Overexpression of ATP-binding cassette (ABC) transporters in cancer cells is associated with the multidrug resistance phenotype (1). Among the 48 human ABC transporters, three have been found predominantly in cell lines selected *in vitro* for drug resistance: P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1), and ABCG2 (1–4). One of the most reproducible detection methods for Pgp and MRP1 has been the measurement of fluorescent dye efflux in flow cytometry-based functional assays. These functional assays have been useful particularly to quantitate expression and function of Pgp and MRP1 in leukemic samples (5). Functional assays for ABCG2 also have been developed for patient samples using the ABCG2 substrates mitoxantrone, BODIPY-prazosin, or topotecan (6–8). However, use of these compounds is limited because they also are substrates of Pgp (6). An ABCG2-specific fluorescent substrate would have direct clinical application in determining the contribution of ABCG2 to clinical drug resistance. In a study of mice lacking Abcg2, Jonker *et al.* (9) noted that mice fed a diet high in alfalfa and housed close to a light source were prone to phototoxic skin lesions. These lesions were believed to be caused by a chlorophyll catabolite, pheophorbide a (PhA), because plasma levels of PhA were 17-fold higher in Abcg2$^{-/-}$ mice than in wild-type mice. Because Jonker *et al.* found PhA to be fluorescent, we wanted to determine whether this compound would prove to be an ABCG2-specific substrate. Additionally, we sought to develop a flow cytometric assay to determine expression and function of ABCG2 using PhA.

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Materials and Methods

Chemicals. Cyclosporin A, verapamil, etoposide, and probenecid were purchased from Sigma Chemical (St. Louis, MO). PhA was obtained from Frontier Scientific (Logan, UT). Flavopiridol and UCN-01 were obtained from the National Cancer Institute Anticancer Drug Screen (Bethesda, MD). Fumitremorgin C (FTC) was isolated by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, NIH (Bethesda, MD). Calcein AM was obtained from Molecular Probes (Eugene, OR). Tariquidar (XR9576) was a gift of Xenova Research (Slough, Berkshire, United Kingdom).

Cell Lines. Selected cell lines overexpressing Pgp, MRP1, or ABCG2 were examined and are listed in Table 1. All of the cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotic, except for MCF-7 cells and sublines, which were maintained in Improved Minimum Essential Medium (IMEM). We also examined HEK-293 cells that were transfected with empty pcDNA3 vector (Invitrogen, Carlsbad, CA) or pcDNA3 vector containing full-length *ABCG2* encoding arginine, threonine, or glycine at amino acid 482. ABCG2 transfectants were grown in Eagle's Minimum Essential Medium (EMEM; American Type Culture Collection, Manassas, VA) supplemented with 10% FCS, glutamine, and antibiotic along with 2 mg/ml G418 (Invitrogen). The transfected cells have been characterized previously (10).

Immunoblot Analysis. Microsomal membrane protein (30 μg) was loaded onto a premade 8% (w/v) SDS-polyacrylamide gel, subjected to electrophoresis, and electrotransferred onto nitrocellulose membranes. Blots were probed sequentially with the monoclonal anti-MRP1 antibody MRPm2 (Kamiya Biomedical, Seattle, WA), the anti-Pgp antibody C219 (Signet Laboratories, Dedham, MA), and the anti-ABCG2 antibody BXP-21 (Kamiya Biomedical).

Flow Cytometry. The flow cytometry studies presented here are based on those described previously (6). Briefly, trypsinized cells were incubated in complete medium (phenol red-free IMEM with 10% FCS) with 10 μM PhA with or without 10 μM of the ABCG2 inhibitor FTC for 30 min at 37°C in 5% CO_2. Cells were washed subsequently with cold complete medium and then incubated for 1 h at 37°C in PhA-free complete medium with 10 μM FTC to generate the FTC/Efflux histogram, or without FTC to generate the Efflux histogram. The cells subsequently were washed twice with cold PBS and analyzed by flow cytometry. FTC/Efflux − Efflux values, the difference in mean channel numbers between the FTC/Efflux and Efflux histograms, were calculated for each cell line as a measure of FTC-inhibitable PhA efflux. For comparison, ABCG2-mediated transport also was assessed using 20 μM mitoxantrone with 10 μM FTC. When assessing Pgp transport, rhodamine 123 (0.5 μg/ml) was used as a substrate, and valspodar (3 μg/ml) was used as the inhibitor; for MRP1 transport, calcein AM (0.5 μM) was used as the substrate, and probenecid (0.5 mM) was used as the inhibitor. When screening potential ABCG2 inhibitors using ABCG2-transfected cells, PhA was used at a concentration of 1 μM with or without the desired inhibitor [5 μg/ml valspodar (PSC-833), 10 μg/ml verapamil, 10 μM cyclosporin A, 50 μM etoposide, 1 μM tariquidar, 100 μM UCN-01, and 100 μM flavopiridol] and 10 μM FTC to generate Inhibitor/Efflux peaks. Results are representative of at least two separate experiments.

In studies with the anti-ABCG2 antibody 5D3, cells were incubated in 2% BSA/DPBS with either phycoerythrin-labeled negative control antibody (IgG2b) or phycoerythrin-labeled 5D3 antibody (both from eBioscience, San Diego, CA) according to the manufacturer's instructions, washed with Dulbecco's Phosphate Buffered Saline (DPBS), and subsequently analyzed. Surface expression of ABCG2 was calculated as the difference in mean channel numbers between the 5D3 histogram and the negative control antibody histo-

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Table 1 *Selected cell lines examined in this study*

Cell line	Selecting drug	Transporter
MCF-7[a]	—	—
MCF-7 MX100[a]	100 nM mitoxantrone	ABCG2 (482R)
MCF-7 FLV100[a]	100 nM flavopiridol	ABCG2 (482R)
MCF-7 FLV250[a]	250 nM flavopiridol	ABCG2 (482R)
MCF-7 FLV500[a]	500 nM flavopiridol	ABCG2 (482R)
MCF-7 FLV1000[a]	1000 nM flavopiridol	ABCG2 (482R)
MCF-7 AdVp10[a]	5 μg/ml verapamil 10 ng/ml Adriamycin	ABCG2 (R482T)
MCF-7 AdVp3000[a]	5 μg/ml verapamil 3000 ng/ml Adriamycin	ABCG2 (R482T)
MCF-7/VP	4 μM etoposide	MRP1[b]
SF295[a]	—	—
SF295 MX20[a]	20 nM mitoxantrone	ABCG2 (482R)
SF295 MX50[a]	50 nM mitoxantrone	ABCG2 (482R)
SF295 MX100[a]	100 nM mitoxantrone	ABCG2 (482R)
SF295 MX250[a]	250 nM mitoxantrone	ABCG2 (482R)
SF295 MX500[a]	500 nM mitoxantrone	ABCG2 (482R)
NCI-H460[a]	—	—
NCI-H460 MX10[a]	10 nM mitoxantrone	ABCG2 (482R)
NCI-H460 MX20[a]	20 nM mitoxantrone	ABCG2 (482R)
S1	—	—
S1-M1-80[a]	80 μM mitoxantrone	ABCG2 (R482G)
SW620	—	—
SW620 Ad300	300 ng/ml Adriamycin	Pgp[c]
M14[a]	—	—
IGROV[a]	—	—

[a] Cells used to correlate fumitremorgin C-inhibitable efflux with surface ABCG2 expression.
[b] MRP1, multidrug resistance-associated protein 1.
[c] Pgp, P-glycoprotein.

gram. Average values for surface expression and FTC-inhibitable efflux were obtained from at least two separate experiments.

Samples were analyzed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA). Phycoerythrin fluorescence was detected with a 488-nm argon laser and a 585-nm bandpass filter, whereas PhA (or mitoxantrone) was detected using a 635-nm red diode laser and a 561-nm filter. Rhodamine 123 and calcein fluorescence was detected with a 488-nm argon laser and a 530-nm bandpass filter. Where noted, PhA fluorescence also was measured with a 488-nm argon laser and 670-nm bandpass filter. At least 10,000 events were collected for all of the flow cytometry studies. By gating on forward *versus* side scatter, debris was eliminated, and dead cells were excluded based on propidium iodide staining.

Cytotoxicity Assays. Cytotoxicity assays were performed as described previously (10) based on the colorimetric assay of Skehan *et al.* (11).

Results

PhA Is an ABCG2-Specific Substrate. To evaluate the specificity of PhA for ABCG2, we conducted efflux studies on selected cell lines overexpressing Pgp, MRP1, or ABCG2. Fig. 1*A* presents an immunoblot analysis of microsomal membrane protein obtained from the cells examined. High levels of ABCG2 were observed in the MCF-7 MX100 cells; overexpression of MRP1 was observed in the MCF-7/VP cells; and high levels of Pgp were detected in the SW620 Ad300 cells. Flow cytometry studies were performed subsequently on the selected cell lines. Cells were incubated for 30 min in the desired fluorescent substrate (20 μM mitoxantrone for ABCG2-overexpressing cells, 0.5 μM calcein AM for MRP1-overexpressing cells, and 0.5 μg/ml rhodamine 123 for Pgp-overexpressing cells) with or without the desired inhibitor (10 μM FTC for ABCG2, 0.5 mM probenecid for MRP1, and 3 μg/ml valspodar for Pgp), washed, and then allowed to efflux in substrate-free media with or without inhibitor. This procedure then was repeated using 10 μM PhA as the substrate. Efflux of mitoxantrone, calcein, or rhodamine (*top row*) was compared with efflux of PhA (*bottom row*) in the selected cell lines as seen in Fig. 1*B*. MCF-7 MX100 cells demonstrated high levels of FTC-inhibitable mitoxantrone efflux and PhA efflux as shown by the difference between the Efflux histogram (*solid line*) and the FTC/Efflux histogram (*dotted line*; first histogram, rows 1 and 2, respectively; Fig. 1*B*). When MRP1-overexpressing MCF-7/VP cells were incubated in calcein AM with (*dotted line*) or without (*solid line*) 0.5 mM of the MRP1 inhibitor probenecid, high levels of probenecid-inhibitable calcein efflux were observed (second histogram, row 1; Fig. 1*B*). However, when MCF-7/VP cells were incubated in PhA with (*dashed line*) or without (*solid line*) probenecid, minimal probenecid-inhibitable PhA efflux was seen (second histogram, row 2; Fig. 1*B*). High levels of valspodar-inhibitable rhodamine 123 efflux were observed in Pgp-overexpressing SW620 Ad300 cells (last histogram, row 1; Fig. 1*B*). In contrast, no valspodar-inhibitable PhA efflux was observed in these cells (last histogram, row 2; Fig. 1*B*). Thus, among the three ABC transporters associated with multidrug resistance, PhA appears to be an ABCG2-specific substrate.

In MCF-7 parental cells, a small but detectable amount of FTC-inhibitable PhA was observed when these cells were incubated in PhA without (*solid line*) or with (*dashed line*) FTC (first histogram; Fig. 1*C*), consistent with earlier observations that MCF-7 parental cells express low levels of ABCG2 (6). A small amount of probenecid-inhibitable PhA efflux also was observed in MCF-7 parental cells (second histogram, row 3; Fig. 1*B*), much like what was observed in the MCF-7/VP cells (second histogram, first row; Fig. 1*C*), suggesting that the slight amount of PhA efflux in the MCF-7/VP cells is not caused by MRP1. Additionally, no valspodar-inhibitable PhA efflux is detected in SW620 parental cells (last histogram; Fig. 1*C*).

Transport of PhA by ABCG2 Is Not Affected by Mutations at Amino Acid 482. Because amino acid 482 has been shown to affect the substrate specificity of the ABCG2 protein, we examined transport of PhA in HEK-293 cells stably transfected with wild-type (482R) or mutant (482T and 482G) ABCG2. Cells that overexpress the wild-type protein (482R) are resistant to mitoxantrone and the camptothecins topotecan and SN-38. When the amino acid at position 482 is mutated to a threonine (482T) or glycine (482G), rhodamine 123 and the anthracyclines are added to the list of substrates (10, 12–14). We selected transfectants that expressed comparable levels of ABCG2 as assessed by staining with 5D3 antibody and performed efflux studies on these cells. All of the transfectants transported PhA as seen in Fig. 1*D*, suggesting transport was unaffected by amino acid 482 mutations.

Correlation of PhA Efflux with ABCG2 Expression. In previous studies with mitoxantrone and BODIPY-prazosin, a linear relationship was found between FTC-inhibitable efflux and expression of ABCG2 mRNA as measured by Northern blot analysis (6). When average values of FTC-inhibitable PhA efflux were plotted against average values of ABCG2 surface expression as determined with the 5D3 antibody in 20 parental and selected cell lines (marked “a” in Table 1), a linear relationship was observed as seen in Fig. 1*E* (*top graph*) with $r^2 = 0.87$.

FTC-inhibitable PhA efflux values were calculated by measuring the fluorescence of PhA with a 635-nm laser and a 561-nm bandpass filter; however, PhA fluorescence also could be detected using a 488-nm argon laser with a 670-nm long pass filter. This also has been reported for mitoxantrone (7). We next calculated FTC-inhibitable PhA efflux values with excitation at 488 nm for selected cell lines and transfectants and compared this value with the surface expression of ABCG2 and again found a linear correlation as seen in Fig. 1*E* (*bottom graph*) with $r^2 = 0.83$.

Screening Potential Inhibitors of ABCG2. We next used PhA efflux as a tool to identify new inhibitors of ABCG2-mediated drug resistance. Because the Pgp inhibitor GF120918 also was shown to be an ABCG2 inhibitor, we examined the ability of the known Pgp inhibitors verapamil, valspodar, cyclosporin A, and tariquidar to inhibit ABCG2-mediated PhA transport (15). The ability of the cyclin-dependent kinase inhibitor UCN-01 to inhibit PhA transport was

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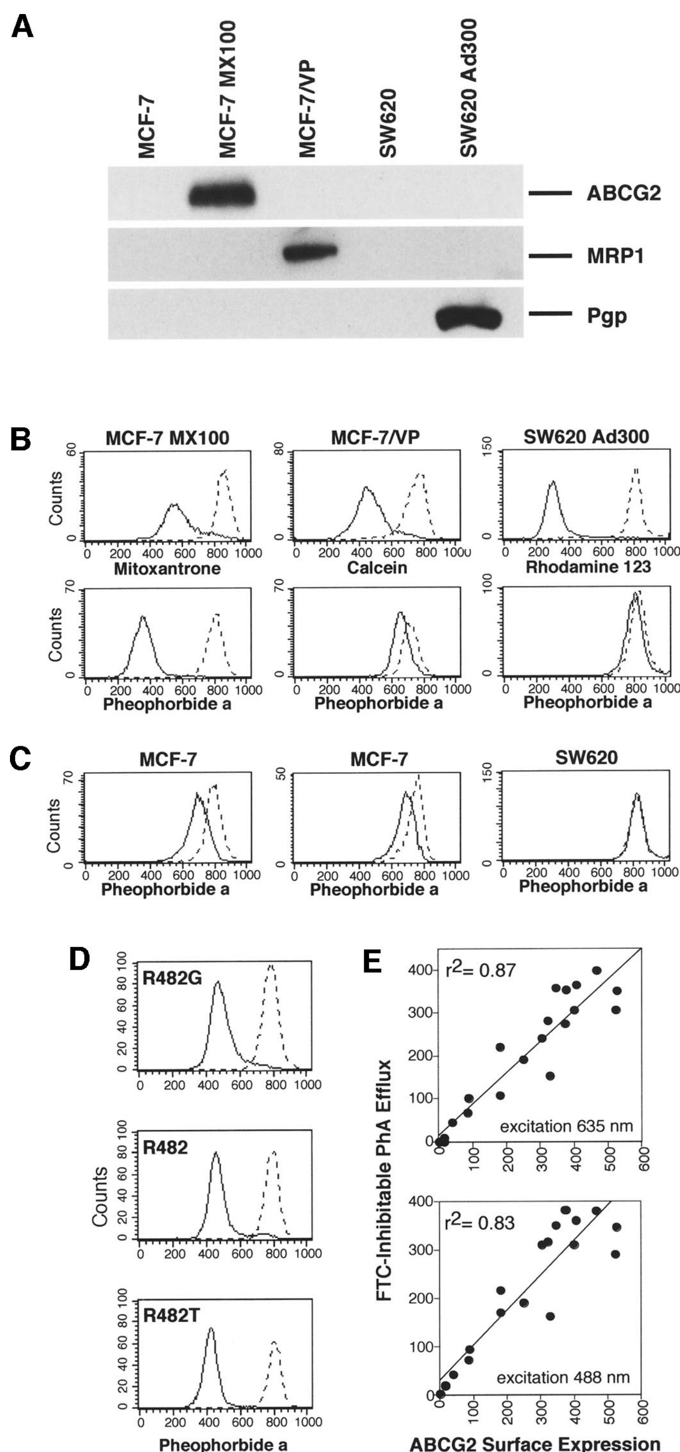

Fig. 1. Pheophorbide a (PhA) is an ABCG2-specific substrate that is unaffected by amino acid 482 mutations. *A,* microsomal membrane protein (30 μg) was electrophoretically separated and transferred to nitrocellulose. Ponceau staining confirmed comparable loading. The blot then was sequentially probed with monoclonal antibodies for P-glycoprotein (*Pgp*; C219), multidrug resistance-associated protein 1 (*MRP1*; MRPm2), and ABCG2 (BXP-21). *B,* selected cell lines overexpressing ABCG2 (MCF-7 MX100), MRP (MCF-7/VP), or Pgp (SW620 Ad300) were incubated with 20 μM mitoxantrone, 0.5 μM calcein AM, or 0.5 μg/ml rhodamine 123, respectively, with or without the desired inhibitor [10 μM fumitremorgin C (*FTC*) for ABCG2, 0.5 mM probenecid for MRP, or 3 μg/ml valspodar for Pgp] for 30 min at 37°C, washed, and then incubated for 60 min in substrate-free media with (*dotted line*) or without (*solid line*) the inhibitor to generate the top row of histograms. The same procedure was repeated with all of the cell lines to generate the second row of histograms except that 10 μM PhA were used as the fluorescent substrate. *C,* MCF-7 parental cells were incubated with 10 μM PhA alone (*solid line*) or with 10 μM FTC (*dashed line*) to generate the first histogram, or cells were incubated with PhA alone (*solid line*) or with 0.5 mM probenecid (*dashed line*) to generate the middle histogram. SW620 cells were incubated with 10 μM PhA without (*solid line*) or with 3 μg/ml valspodar (*dashed line*) to generate the last histogram. *D,* ABCG2-transfected HEK-293 cells expressing comparable levels of wild-type (482R) or mutant (482G and 482T) ABCG2 were incubated in 1 μM PhA with (*dashed line*) or without (*solid line*) 10 μM FTC. *E,* average values for ABCG2 surface expression as determined by 5D3 antibody staining were plotted *versus* average values of FTC-inhibitable PhA efflux for 20 parental and resistant cell lines shown in Table 1. FTC-inhibitable efflux values were obtained by excitation of PhA at 635 nm (*top graph*) or 488 nm (*bottom graph*).

tested based on previous observations that the cyclin-dependent kinase inhibitor flavopiridol inhibits ABCG2 function (16). Etoposide, a relatively poor ABCG2 substrate, also was examined to see whether it might competitively inhibit PhA transport (10). HEK-293 cells transfected with either wild-type or mutant ABCG2 were incubated with PhA in the presence of the potential ABCG2 inhibitor. We included wild-type and mutant ABCG2-transfected cells in our analysis because previous results with novobiocin demonstrated that amino acid 482 can affect inhibitor specificity (10). FTC at a concentration of 10 μM was included as a positive control. Verapamil at 10 μg/ml had no effect on PhA transport (Fig. 2). Surprisingly, the Pgp inhibitor tariquidar was able to inhibit ABCG2-mediated transport at a concentration of 1 μM. UCN-01 also was able to inhibit wild-type and mutant ABCG2 proteins at a concentration of 100 μM. In agreement with previous reports, 100 μM of the cyclin-dependent kinase inhibitor flavopiridol was able to completely inhibit ABCG2 (16). The compounds cyclosporin A (10 μM), etoposide (50 μM), and valspodar (5 μg/ml) did not inhibit ABCG2-mediated PhA transport (data not shown). Representative results with verapamil (10 μg/ml), tariquidar (1 μM), UCN-01 (100 μM), flavopiridol (100 μM), and FTC (10 μM) are shown in Fig. 2.

Tariquidar Inhibits ABCG2-Mediated SN-38 and Topotecan Resistance. To confirm inhibition of ABCG2-mediated transport by tariquidar, we performed 4-day cytotoxicity assays on ABCG2-transfected cells with SN-38 or topotecan in the presence or absence of 1 μM tariquidar. Cytotoxicity assays were conducted with SN-38 or topotecan because previous studies had demonstrated that the ABCG2-transfected cells had comparable IC_{50} values for these compounds (10). The addition of 1 μM tariquidar was able to abrogate almost completely ABCG2-mediated SN-38 or topotecan resistance. Representative results for SN-38 are shown in Fig. 3*A*. Dose-modifying factors also were calculated for each transfectant by dividing the IC_{50} of each drug alone by the IC_{50} of each drug in the presence of tariquidar. The dose-modifying factors for 482R-, 482G-, or 482T-transfected cells with SN-38 were 75, 52, and 40, respectively, and with topotecan were 10, 33, and 13, respectively, indicating substantial resistance reversal of wild-type and mutant ABCG2 proteins.

ABCG2 Confers Resistance to UCN-01. Previous studies with the cyclin-dependent kinase inhibitor flavopiridol showed it to be an inhibitor and substrate of ABCG2 (16). On the basis of these earlier studies, we thought UCN-01 also might be a substrate for ABCG2. In 4-day cytotoxicity assays with UCN-01, cells transfected with either wild-type (482R, *open circles*) or mutant (482G, *hatched squares*; 482T, *filled triangles*) ABCG2 were approximately 6–7-fold resistant to the drug compared with empty vector-transfected cells (*filled circles*); representative results from two separate experiments are shown in Fig. 3*B*.

Discussion

The chlorophyll catabolite PhA, recently shown to be a fluorescent substrate of ABCG2 by Jonker *et al.* (9), was used to develop a flow cytometry-based assay for ABCG2 expression and function. Previous methods for detecting ABCG2 function and expression in clinical samples have centered on the fluorescent compounds mitoxantrone and BODIPY-prazosin (6, 7). However, the use of these compounds

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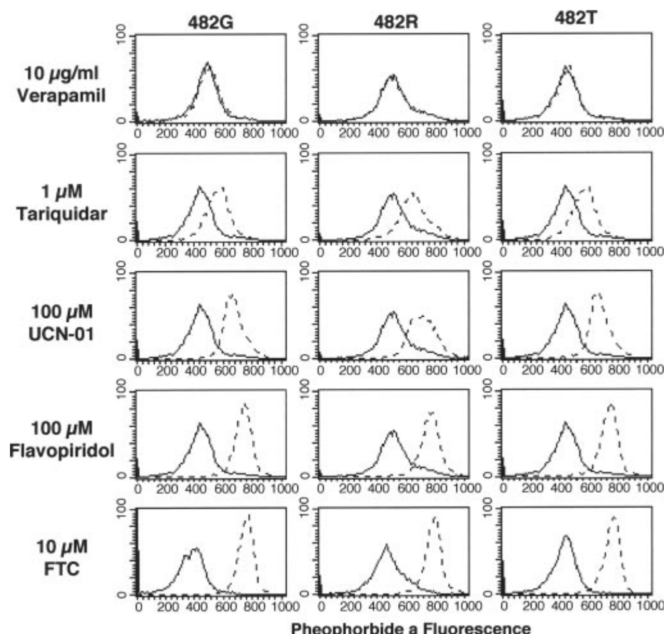

Fig. 2. Screening for inhibitors of ABCG2. HEK-293 cells transfected with wild-type (482R) or mutant (482G and 482T) ABCG2 were incubated in 1 μM pheophorbide a alone (*solid line*) or with various inhibitors (*dotted line*). Representative results are shown for verapamil, tariquidar, UCN-01, flavopiridol, and fumitremorgin C (*FTC*).

is compromised by their transport by Pgp (6). Among drug-selected cell lines overexpressing Pgp, MRP, or ABCG2, PhA appears to be a substrate of ABCG2 alone, potentially allowing specific detection of ABCG2 even in clinical samples that may express MRP1 or Pgp in addition to ABCG2. To our knowledge, this is the first report of a fluorescent substrate that can discriminate ABCG2. These results suggest that PhA transport may form the basis of a sensitive and specific assay for ABCG2 in the clinical setting.

FTC-inhibitable PhA transport was found to correlate well with ABCG2 expression in selected cell lines overexpressing ABCG2. As also has been described for mitoxantrone, PhA fluorescence was detected by excitation with a standard 488-nm argon laser and a 635-nm red diode laser, allowing detection of PhA fluorescence on standard flow cytometers (7). This property may be particularly helpful to isolate "side population" cells. Side population cells have been isolated from many tissue types, but isolation of these cells involves the use of Hoechst 33342, a fluorescent dye that requires UV excitation. Expression of ABCG2 and Pgp has been demonstrated in bone marrow stem cell populations, and it is believed that ABCG2 expression may define primitive quiescent hematopoietic stem cells, whereas Pgp may be expressed in more activated repopulating stem cells (17). Hoechst 33342 is a substrate of Pgp and ABCG2; therefore, activated cells that express Pgp will be isolated with more primitive cells that express ABCG2. Thus, a potential use of PhA is to isolate primitive ABCG2-expressing stem cells.

Because there is some overlap in substrate specificity between ABCG2 and Pgp, we evaluated the ability of known Pgp inhibitors to abrogate ABCG2-mediated PhA transport. Our results show tariquidar (XR9576) to be a multispecific blocker as has been described for GF120198 (15). Although tariquidar inhibits Pgp at nanomolar concentrations, micromolar concentrations are needed to inhibit ABCG2, likely indicating a higher affinity for Pgp than for ABCG2 (18). Therefore, tariquidar, as with GF120918, would be expected to increase the oral bioavailability of topotecan (19). It is not known whether tariquidar itself is transported by ABCG2. Ultimately, the value of ABCG2 inhibitors or multispecific inhibitors will depend on the importance of the drug transporters in clinical drug resistance. Proof of this for Pgp has been difficult to attain despite numerous clinical trials attempting its modulation (20).

We have demonstrated previously overexpression of ABCG2 in cells selected for resistance to the cyclin-dependent kinase inhibitor flavopiridol, and inhibition of ABCG2-mediated transport by flavopiridol (16). Our previous findings led us to test the ability of the cyclin-dependent kinase inhibitor UCN-01 to inhibit PhA transport in ABCG2-transfected cells. Transport of PhA was inhibited by 100 μM UCN-01. Because flavopiridol was found to be an inhibitor and a substrate of ABCG2, 4-day cytotoxicity assays were performed with UCN-01 on ABCG2-transfected cells. ABCG2 was shown to confer resistance to UCN-01, most likely indicating that UCN-01 is a substrate of ABCG2 and therefore most likely a competitive inhibitor of ABCG2-mediated transport. The results presented here suggest that the effectiveness of UCN-01 may be limited in tumors that overexpress ABCG2.

Amino acid 482 has been shown to play a pivotal role in the substrate and inhibitor specificity of ABCG2. Initial studies of drug-selected cell lines overexpressing ABCG2 often demonstrated discrepancies in their cross-resistance profiles regarding anthracycline resistance, and later studies linked these discrepancies to a difference in the amino acid at position 482 (21, 22). The ability of amino acid 482 to affect the substrate specificity of ABCG2 was confirmed later in transient transfection assays (12). Stable transfectants expressing wild-type ABCG2 with an arginine at amino acid 482 were resistant to mitoxantrone, SN-38, and camptothecin, whereas cells transfected with mutant ABCG2 with a glycine or threonine at position 482 also were resistant to the anthracyclines and rhodamine 123 (10). PhA was found to be transported by wild-type and mutant ABCG2, as has been shown for mitoxantrone. Allen *et al.* (14) have postulated that the loss of the negatively charged arginine may be a factor to determine the substrate specificity of ABCG2, suggesting that the wild-type protein would not transport the positively charged compounds. However, PhA is predicted to carry a net positive charge at physiologic pH, much like

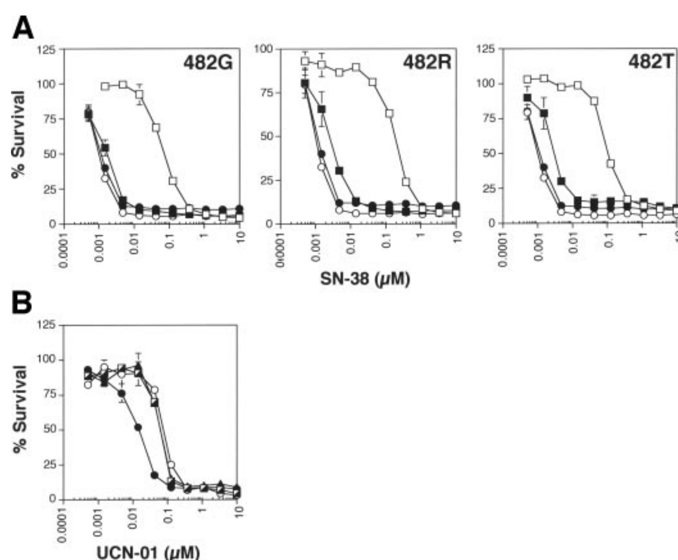

Fig. 3. Interaction of tariquidar and UCN-01 with ABCG2. *A,* 4-day cytotoxicity assays were conducted on empty vector-transfected HEK-293 cells (● and ○) or HEK-293 cells transfected with wild-type (482R) or mutant (482G and 482T) ABCG2 (■ and □) using SN-38 or topotecan with (● and ■) or without (○ and □) 1 μM tariquidar. Representative results from two independent experiments are shown. *B,* 4-day cytotoxicity assays were performed with UCN-01 on HEK-293 cells transfected with wild-type 482R (○) or mutant 482G (◪) and 482T ABCG2 (▲). HEK-293 cells transfected with empty vector (●) are shown for comparison. Representative results from two independent experiments are shown.

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mitoxantrone, which is a substrate for wild-type and mutant ABCG2. Thus, the role of amino acid 482 in substrate specificity remains to be elucidated.

In summary, PhA has been shown to be an ABCG2-specific substrate, and FTC-inhibitable PhA efflux correlates with ABCG2 expression. FTC-inhibitable efflux is detectable in cells that express low amounts of the protein, making this assay useful to determine ABCG2 expression and function in clinical samples. Additional studies exploring the potential of tariquidar as an ABCG2 inhibitor are warranted.

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