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Identification of Compounds that Correlate with ABCG2 Transporter Function in the National Cancer Institute Anticancer Drug Screen[S]

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ABSTRACT

ABCG2 is an ATP-binding cassette transporter that counts multiple anticancer compounds among its substrates and is believed to regulate oral bioavailability as well as serve a protective role in the blood-brain barrier, the maternal-fetal barrier, and hematopoietic stem cells. We sought to determine whether novel compounds that interact with the transporter could be identified through analysis of cytotoxicity profiles recorded in the NCI Anticancer Drug Screen database. A flow cytometric assay was used to measure ABCG2 function in the 60 cell lines and generate a molecular profile for COMPARE analysis. This strategy identified >70 compounds with Pearson correlation coefficients (PCCs) >0.4, where reduced drug sensitivity correlated with ABCG2 expression, as well as >120 compounds with PCCs < −0.4, indicating compounds to which ABCG2 expression conferred greater sensitivity. Despite identification of known single nucleotide polymorphisms in the *ABCG2* gene in a number of the cell lines, omission of these lines from the COMPARE analysis did not affect PCCs. Available compounds were subjected to validation studies to confirm interaction with the transporter, including flow cytometry, [^{125}I]IAAP binding, and cytotoxicity assays, and interaction was documented in 20 of the 27 compounds studied. Although known substrates of ABCG2 such as mitoxantrone or topotecan were not identified, we characterized three novel substrates—5-hydroxypicolinaldehyde thiosemicarbazone (NSC107392), (*E*)-*N*-(1-decylsulfanyl-3-hydroxypropan-2-yl)-3-(6-methyl-2,4-dioxo-1*H*-pyrimidin-5-yl)prop-2-enamide (NSC265473), and 1,2,3,4,7-pentahydroxy-1,3,4,4*a*,5,11*b*-hexahydro[1,3]dioxolo[4,5-*j*]phenanthridin-6(2*H*)-one [NSC349156 (pancratistatin)]—and four compounds that inhibited transporter function—2-[methyl(2-pyridin-2-ylethyl)-amino]fluoren-9-one hydroiodide (NSC24048), 5-amino-6-(7-amino-5,8-dihydro-6-methoxy-5,8-dioxo-2-quinolinyl)-4-(2-hydroxy-3,4-dimethoxyphenyl)-3-methyl-2-pyridinecarboxylic acid, methyl ester (NSC45384), (17β)-2,4-dibromo-estra-1,3,5(10)-triene-3,17-diol (NSC103054), and methyl *N*-(pyridine-4-carbonylamino)carbamodithioate (NSC636795). In summary, COMPARE analysis of the NCI drug screen database using the ABCG2 functional profile was able to identify novel substrates and transporter-interacting compounds.

ABCG2 is an ATP-binding cassette (ABC) half-transporter that has been linked to chemotherapeutic drug resistance. ABCG2 is highly expressed in a variety of normal tissues, including the endothelium in the central nervous system and the placenta as well as the small intestine, liver, and bile canniculi (Maliepaard et al., 2001; Fetsch et al., 2005). These expression levels and localizations highlight the likely role of ABCG2 in contributing to the blood-brain barrier, maternal-fetal barrier, and blood-germ cell barrier as well as its role in the absorption and efflux of xenobiotics (Deeken and Löscher, 2007; Hardwick et al., 2007; Robey et al., 2007). In addition, ABCG2 is highly expressed in a number of cancers, including lung, endometrial, and gastrointestinal malignancies (Robey et al., 2007). Several studies have pointed to a role for ABCG2 in drug resistance in acute myelogenous leukemia (Benderra et al., 2004; Suvannasankha et al., 2004).

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A number of chemotherapeutic agents are substrates for the transporter, including mitoxantrone, topotecan, irinotecan and its active metabolite SN-38, methotrexate, flavopiridol, and some tyrosine kinase inhibitors (Hardwick et al., 2007; Robey et al., 2007). Several compounds have also been reported to act as inhibitors of ABCG2, including Fumitremorgin C (FTC) (Rabindran et al., 1998); the P-glycoprotein inhibitors elacridar (GF 120918; de Bruin et al., 1999), tariquidar (XR9576; Robey et al., 2004) and biricodar (VX-710; Minderman et al., 2004); and the tyrosine kinase inhibitors imatinib, nilotinib, and gefitinib (Robey et al., 2007). ABCG2 inhibitors have potential uses in increasing oral bioavailability or CNS penetration of substrate drugs, potentially leading to more effective cancer treatments.

Somatic single-nucleotide polymorphisms, or SNPs, have been discovered in the *ABCG2* gene (Honjo et al., 2002; Zamber et al., 2003). One nonsynonymous substitution, 421C>A (dbSNP 914C>A, rs2231142), leads to an amino acid substitution of lysine for glutamine at position 141 and has been shown to result in lower plasma membrane expression, reduced drug efflux, and reduced ATPase activity (Imai et al., 2002; Mizuarai et al., 2004; Morisaki et al., 2005). The SNP also has been found to correlate with higher drug levels in vivo in patients exposed to chemotherapy drugs (Hardwick et al., 2007; Robey et al., 2007). This allelic variant has a broad range in frequency across different ethnic populations, with low frequency in African Americans (2–5%), moderate frequency in people of European (11–14%), Hispanic (10%), and Middle Eastern (13%) descent, and high frequency in people of Chinese (35%) and Japanese (35%) descent (Lepper et al., 2005). Although other SNPs within the *ABCG2* gene have not been found to correlate with a change in function or pharmacokinetics in vivo, Rudin et al. (2008) correlated a SNP within the first intron of the gene (rs2282622) with increased toxicity in patients treated with erlotinib.

The National Cancer Institute (NCI) Developmental Therapeutics Program (DTP)'s Anticancer Drug Screen (NCI-ADS) has been used successfully over the past 2 decades to identify and classify new cancer therapies. Cytotoxicity assays have been performed on more than 140,000 compounds against a set of 60 cell lines from various malignancies. The COMPARE program is a web-based tool configured to calculate pair-wise correlations between these cytotoxicity patterns, or "fingerprints," and other profiles, whether they be other cytotoxicity profiles or expression profiles of molecular targets within the cell lines. Targets such as the epidermal growth factor receptor, erbB2, p53, and Chk2 have been studied using this tool (O'Connor et al., 1997; Wosikowski et al., 1997; Jobson et al., 2007; Liu et al., 2007). This method has also been used to study ABC transporters. Both expression and function profiles of P-glycoprotein (P-gp, ABCB1) were used to identify P-gp substrates and inhibitors through correlating cytotoxicity patterns with the P-gp data (Lee et al., 1994; Alvarez et al., 1995). Likewise, expression and function of MRP1 (ABCC1) was also used to probe the NCI-ADS for possible transporter substrates (Alvarez et al., 1998). In contrast to P-gP, MRP1 expression and function were poor predictors of MRP1 substrates or inhibitors (Alvarez et al., 1998).

ABCG2 mRNA expression in the 60 cell lines has previously been measured and used as the seed for a COMPARE analysis; however, a significant correlation between ABCG2 expression and patterns of drug sensitivity did not emerge (Szakács et al., 2004), and no substrates or inhibitors of ABCG2 were identified. In this study, we used an assay based on pheophorbide A (PhA) efflux that measured ABCG2 transporter activity and thereby function. The functional data were used as the seed in a COMPARE analysis to investigate possible correlations with the drug sensitivity and resistance patterns of compounds contained in the NCI-ADS.

Materials and Methods

Materials. Compounds used for this study were obtained through the DTP of the NCI (Bethesda, MD). PhA was obtained from Frontier Scientific (Logan, UT). FTC was prepared by Thomas McCloud (Screening Technologies Branch, DTP, NCI). Topotecan was purchased from LKT laboratories (St. Paul, MN). [^{125}I]iodoarylazidoprazosin (IAAP) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell Lines and Cell Culture. The cell lines of the NCI Anticancer Drug Screen panel were obtained and grown in monolayers or in suspension in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) stably trans-

ABBREVIATIONS: ABC, ATP-binding cassette; SN-38, 7-ethyl-10-hydroxycamptothecin glucuronide; FTC, Fumitremorgin C; GF 120918, elacridar; XR9576, tariquidar; VX-710, biricodar; SNP, single nucleotide polymorphism; NCI-ADS, National Cancer Institute Developmental Therapeutics Program's Anticancer Drug Screen; DTP, Developmental Therapeutics Program; P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; PhA, pheophorbide A; IAAP, iodoarylazidoprazosin; HEK, human embryonic kidney; PCC, Pearson correlation coefficient; NSC103054, (17β)-2,4-dibromo-estra-1,3,5(10)-triene-3,17-diol; NSC107392, 5-hydroxypicolinaldehyde thiosemicarbazone; NSC114609, (6*E*)-6-[[(1,3-dihydroxy-2-methylpropan-2-yl)amino]methylidene]-2,4-diiodocyclohexa-2,4-dien-1-one; NSC153330, 2-methyl-5,6,7,8-tetrahydro-3*H*-[1]benzothiolo[2,3-*d*]pyrimidin-4-one; NSC174939, 1-*N*′,3-*N*′-bis(benzenecarbonothioyl)-1-*N*′,3-*N*′-dimethylpropanedihydrazide; NSC24048, 2-[methyl(2-pyridin-2-ylethyl)amino]fluoren-9-one hydroiodide; NSC265473/NSC305458, (*E*)-*N*-(1-decylsulfanyl-3-hydroxypropan-2-yl)-3-(6-methyl-2,4-dioxo-1*H*-pyrimidin-5-yl)prop-2-enamide; NSC297093, 2,2-dimethyl-5-[(5-nitrothiophen-2-yl)methylidene]-1,3-dioxane-4,6-dione; NSC349156, 1,2,3,4,7-pentahydroxy-1,3,4,4*a*,5,11*b*-hexahydro[1,3]dioxolo[4,5-*j*]phenanthridin-6(2*H*)-one (pancratistatin); NSC382054, 6-ethyl-5-(3-oxo-2-phenyl-1*H*-benzotriazol-3-ium-5-yl)pyrimidine-2,4-diamine; NSC45384, 5-amino-6-(7-amino-5,8-dihydro-6-methoxy-5,8-dioxo-2-quinolinyl)-4-(2-hydroxy-3,4-dimethoxyphenyl)-3-methyl-2-pyridinecarboxylic acid, methyl ester; NSC608001, 4-[(5,5,8,8-tetramethyl-6,7-dihydronaphthalene-2-carbonyl)amino]benzoic acid; NSC620303, *N*′-[(*E*)-(3-iodo-5-nitro-6-oxocyclohexa-2,4-dien-1-ylidene)methyl]-3-nitrobenzenesulfonohydrazide; NSC620515, 2-chloro-8-hydroxy-6-methoxy-7-methylnaphthalene-1,4-dione; NSC623636, 2-bromo-5-methoxycyclohexa-2,5-diene-1,4-dione; NSC625546, [4-(2,4-dihydroxybenzoyl)-2,5-bis(4-hydroxyphenyl)oxolan-3-yl]-(2,4-dihydroxyphenyl)methanone; NSC636795, methyl *N*-(pyridine-4-carbonylamino)carbamodithioate; NSC651644, 4-amino-*N*-[3-chloro-2-(3-nitrophenyl)-4-oxoazetidin-1-yl]benzamide; NSC686342, 2-[3-cyano-5,5-dimethyl-4-[(*E*)-2-pyridin-4-ylethenyl]furan-2-ylidene]propanedinitrile; NSC691417, ethyl (1*E*)-*N*-[5-cyano-4-(3,4-dimethoxyphenyl)-1,3-diphenyl-4*H*-pyrano[2,3-*c*]pyrazol-6-yl]methanimidate; NSC722812, 5-[(5-cyano-2-hydroxy-6-morpholin-4-yl-4-oxo-1*H*-pyridin-3-yl)-(2,6-dichlorophenyl)methyl]-6-hydroxy-2-morpholin-4-yl-4-oxo-1*H*-pyridine-3-carbonitril.

fected with empty pcDNA 3.1 vector (Invitrogen, Carlstad, CA) (pcDNA) or vector containing full-length *ABCG2* R-2 were maintained in Earle's minimal essential medium supplemented with 2 mg/ml G418 to enforce transporter expression (Robey et al., 2003). Validation studies also used ABCG2-overexpressing NCI-H460 MX20 cells that were obtained by step-wise selection and were maintained in 20 nM mitoxantrone (Robey et al., 2004). ABCG2-overexpressing MCF-7 FLV1000 cells were maintained in Richter's medium with 1000 nM flavopiridol (Robey et al., 2001). DNA isolated from the cell lines of the NCI drug screen was provided by the NCI DTP.

ABCG2 Functional Assay. Functional assays with PhA were performed as described previously with minor modifications (Robey et al., 2004). In brief, trypsinized cells were incubated for 30 min at 37°C in 1 μM PhA in the presence or absence of 10 μM FTC, an ABCG2 inhibitor. The cells were subsequently washed and incubated at 37°C for 1 h in PhA-free medium continuing with FTC to block ABCG2-mediated efflux of PhA and generate the FTC/efflux histogram (Fig. 1A, dashed line) or continuing without FTC during the 1-h efflux period, generating the efflux histogram (solid line). PhA fluorescence was measured on a FACSort flow cytometer equipped with a 635-nm red diode laser. The difference in mean channel number between the FTC/efflux and efflux histogram, termed the inhibitable efflux, was calculated, and each cell line was tested at least twice. This value has been previously shown to correlate with ABCG2 expression (Robey et al., 2004). When the inhibitable efflux value was negative, it was assigned the value of zero.

This same method was used to test whether compounds identified in the NCI-ADS as potentially interacting with ABCG2 could inhibit transporter function. Potentially interacting compounds were incubated with wild-type ABCG2 transfected cells at a concentration of 10 μM to determine whether they inhibited PhA efflux. -Fold increase in PhA fluorescence was obtained by dividing the intracellular PhA fluorescence in the presence of each compound by the fluorescence in the absence of compound. Each compound was tested at least twice.

ABCG2 Expression Data. Measures of *ABCG2* gene expression were obtained from the NCI DTP. These results, obtained by polymerase chain reaction (Szakács et al., 2004) and gene expression microarrays (Lee et al., 2003), are publicly available (http://dtp.nci.nih.gov/mtargets/mt_index.html). Seven measures of *ABCG2* mRNA were available and included in this study (NCI DTP identification numbers MT2678, GC14733, GC36729, GC56458, GC93477, GC152721, and GC228107).

ABCG2 SNP Genotyping. The NCI 60 cell lines were genotyped for variations in the *ABCG2* gene using DNA provided by the NCI DTP. After double stranded DNA content for each cell line was determined using the Quant-iT Picogreen dsDNA assay kit (Invitrogen, Carlsbad, CA) in conjunction with a FLUOstar Optima (BMG Labtech, Durham, NC) fluorescence plate reader, the identification of SNPs was performed using two different methods. First, genotype identification for seven variant sites in the *ABCG2* gene was done for each cell line using the Affymetrix DMET platform as described previously (Dumaual et al., 2007). Second, genotyping for variations in intron 1 (rs2622604) was performed as follows: 20 ng of genomic DNA was concentrated, applied, and dried to a 384-well thermoplate

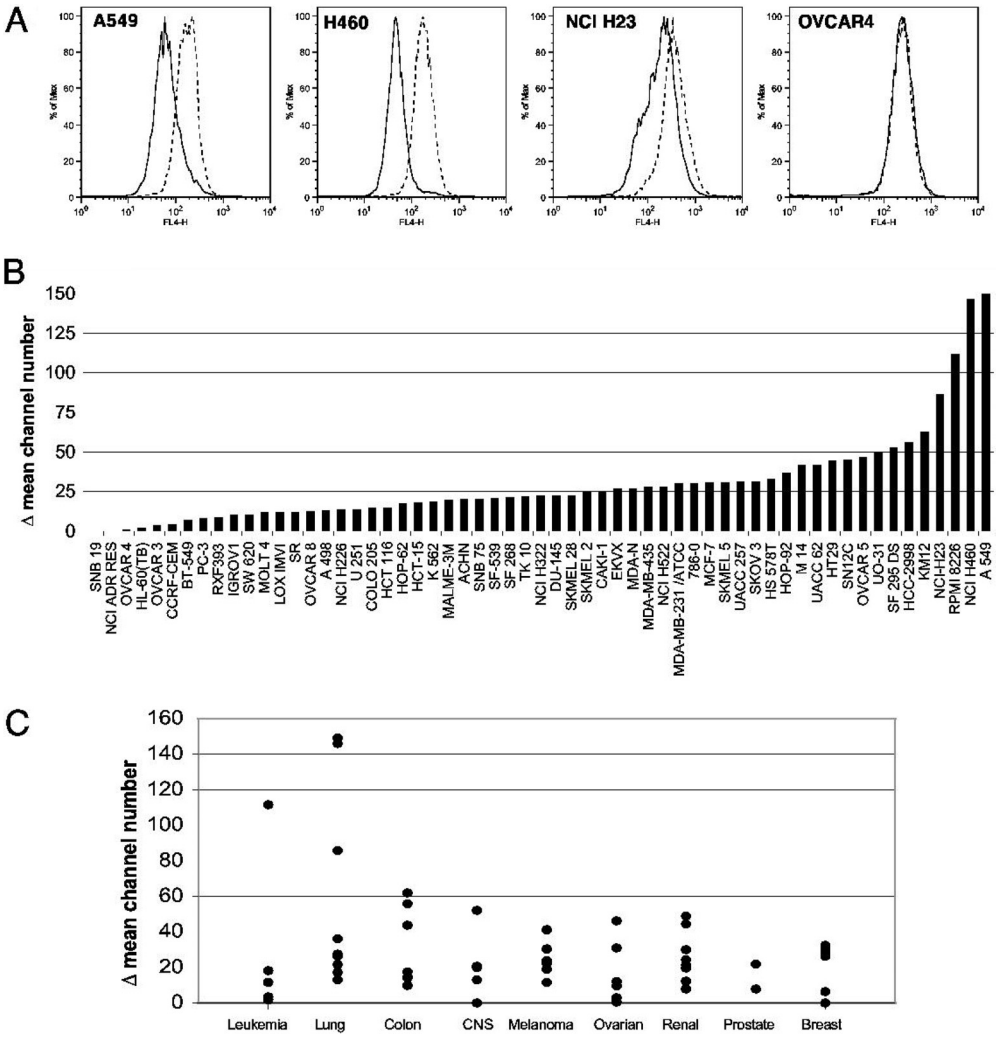

Fig. 1. Pheophorbide A efflux in cell lines from the NCI-ADS. A, cells were trypsinized and incubated in 1 μM PhA in the presence or absence of 10 μM FTC, an ABCG2 inhibitor, for 30 min. Cells were then washed and allowed to incubate for 1 h in PhA-free medium, continuing with (dashed line) or without (solid line) FTC, and intracellular fluorescence of PhA was determined. Representative results from one of at least three experiments with A549, H460, NCI-H23, and OVCAR 4 cells are shown. B, ABCG2 function in the 60 cell lines of the NCI-ADS was measured using PhA as outlined in A above and the difference in mean channel number between the dashed and solid histograms was calculated to obtain a measure of ABCG2-mediated efflux. Bars represent average efflux values from at least three independent experiments for each cell line. C, average efflux values obtained as outlined in B above were plotted versus tumor type.

(Thermo Fisher Scientific, Waltham, MA). Validated SNP identification primers for this variant along with VIC and 5-carboxyfluorescein probes and master mix for the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) were added to the wells, then sealed and processed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) for 40 cycles (95°C for 15 s followed by 60°C for 60 s). SNP classification was performed with the onboard SDS 2.1 software and checked manually to ensure accuracy. The experiment was run in duplicate to confirm results.

National Cancer Institute Drug Screen Database and COMPARE Analysis. The NCI Drug Screen database contains information on more than 140,000 compounds characterized for cytotoxicity patterns in the 60 human cancer cell lines. Cytotoxicity curves are generated, from which the GI_{50} can be determined. The GI_{50} is the time 0-corrected IC_{50} value and is defined as the concentration of an agent that causes 50% growth inhibition. The GI_{50} is different in each cell line, and the difference of that value from the mean GI_{50} for the 60 cell lines for any given compound defines a cytotoxicity pattern that can be displayed as a "mean graph" or "fingerprint" (See Fig. 4). A vertical line represents the mean response of the cell lines to the test agent. The COMPARE database stores the screening data as the $-\log(GI_{50})$ for historical reasons related to the mean graph sign conventions (Lee et al., 1994). This means that data with higher IC_{50} values from more drug-resistant cell lines are stored with smaller (more negative) values and are found graphically to the left of the mean. Drug-sensitive cell lines are graphed to the right. A profile or seed probe can be entered to query the database for compounds that have a high positive or negative correlation. We generated a fingerprint of ABCG2 function in the 60 cell lines of the drug screen and used this as a seed to obtain compounds that have cytotoxicity profiles correlating with the ABCG2 function profile. These compounds were then subjected to further testing.

Competition of [^{125}I]IAAP Labeling. Crude membranes (1 mg of protein/ml) from the MCF-7 FLV1000 cells were incubated in 50 mM Tris-HCl, pH 7.5, containing 20 μM test compound or FTC for 10 min at room temperature. Subsequently, 3 to 6 nM [^{125}I]IAAP (2200 Ci/mmol) was added, and the samples were incubated for an additional 5 min under subdued light. The samples were illuminated with a UV lamp (365 nm) for 10 min at room temperature (21–23°C). Labeled ABCG2 was immunoprecipitated by adding 800 μl of radioimmunoprecipitation assay buffer with 1% aprotinin followed by the addition of 10 μg of BXP-21 antibody (Kamiya Biomedical, Seattle, WA), after which the samples were incubated for 3 h at 4°C. The beads were pelleted by centrifuging at 13,000 rpm for 5 min at 4°C and then washed with radioimmunoprecipitation assay buffer in 1% aprotinin. SDS-PAGE sample buffer (25 μl) was then added, and the samples were incubated for 1 h at 37°C, followed by the addition of 25 μl of water and an additional incubation at 37°C for 30 min. Samples were separated by PAGE on a 7% Tris-acetate gel at constant voltage. The gel was dried and exposed to Bio-Max MR film (Carestream Health, Rochester, NY) for 3 to 6 days at −80°C. The incorporation of [^{125}I]IAAP into the ABCG2 band was quantified by estimating the radioactivity of this band using the STORM 860 PhosphorImager system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and ImageQuaNT software (Shukla et al., 2006).

Cytotoxicity Assays. Four-day cytotoxicity assays with sulforhodamine B were performed as described previously (Skehan et al., 1990). The cells were plated in flat-bottomed 96-well plates (10^4 cells per well for transfected HEK 293 cells and 5×10^3 cells per well for NCI-H460 parental and MX20-resistant cells) and allowed to attach for 24 h at 37°C. Compounds at various concentrations were added to the cells and the plates were allowed to incubate for 96 h at 37°C. The cells were subsequently fixed in 50% trichloroacetic acid and stained with sulforhodamine B solution (0.4% sulforhodamine B in 1% acetic acid). Optical densities were read on a plate reader (Bio-Rad Laboratories, Hercules, CA) at an absorbance of 540 nm. Each concentration was tested in quadruplicate. Combination studies with putative potential ABCG2 inhibitors and topotecan, a known ABCG2 substrate, were performed in wild-type *ABCG2*-transfected and empty vector-transfected cells using this same method. Relative resistance values were calculated by dividing the IC_{50} of the ABCG2-expressing line by its corresponding parental line.

Results

ABCG2 Function and the NCI-60 Cell Lines. Relative ABCG2 transporter function in 59 of the 60 cell lines of the drug screen was measured by flow cytometry using the PhA assay. Cells were incubated with PhA in the absence of FTC to generate the efflux histogram (solid line), whereas cells were incubated in PhA in the presence of FTC to generate the FTC/efflux histogram (dashed line) as outlined under *Materials and Methods* (Fig. 1A). The difference between the two histograms has been shown to correlate with levels of cell surface ABCG2 expression (Robey et al., 2004). Figure 1A shows results from two cell lines found to have the highest levels of ABCG2 function among cells in the screen (A549 and NCI-H460), one cell line with moderate function (NCI-H23), and one with nearly undetectable ABCG2 function (OVCAR4).

Figure 1B summarizes the results from 59 of the 60 cell lines, expressed as the difference in mean channel number between the FTC/efflux and the efflux histograms. This difference reflects the degree of ABCG2 transporter-mediated efflux and thus is a measure of relative ABCG2 function in each cell line. Although levels of ABCG2-mediated PhA efflux were relatively low compared with levels observed in drug-selected cell lines, the highest levels of ABCG2 function were found in the NCI-H460, A549, RPMI-8226, NCI-H23, HCC2998, KM12, and SF295 cell lines. Lower levels were found in IGROV1 and LOX-IMVI, and ABCG2 function was nearly undetectable in HL-60, OVCAR4, and SNB-19 cells.

The NCI-60 cell lines represent nine different tumor types, including leukemia, non–small-cell lung cancer, colon cancer, central nervous system tumors, melanoma, ovarian carcinoma, renal cancer, prostate cancer, and breast cancer. Relative ABCG2 function by tissue type within the DTP cell lines is shown in Fig. 1C. With the exception of four cell lines among the leukemia (RPMI-8226) and lung cancer subsets (A549, NCI-H460, and NCI-H23), the range of ABCG2 function was low across all tumor types.

Correlation between Expression and Function. Various measures of *ABCG2* mRNA available from the NCI Molecular Targets Database were used to determine a correlation between expression and ABCG2 function (MT2888). *ABCG2* mRNA expression was analyzed by reverse transcription-polymerase chain reaction (MT2678) as well as in gene expression arrays, including the Affymetrix U95 (GC36729, GC56458, and GC93477), the Affymetrix U133 (GC152721 and GC228107), and a customized oligonucleotide expression array (GC14733). We constructed a correlation matrix to determine the relationships between ABCG2 function, ABCG2 mRNA expression, and *ABCG2* expression determined by the gene expression arrays (Supplemental Table 1). Pearson correlation coefficients between functional data and expression data ranged from 0.42 (GC14733) to 0.89 (GC228107), suggesting consensus between arrays and the functional data [except for the Affymetrix U95 A chip (GC94377)]. Consensus with the latter and any of the other

variables was low (correlation coefficient of −0.05 to 0.14), but data from this chip are generally considered unreliable.

SNPs in the NCI-60 Cell Lines. Because SNP variants are known to impair ABCG2 expression and function, cells expressing these variants may confound results based on mRNA expression. Thus, we evaluated *ABCG2* genotypes in the cell lines of the drug screen. DNA from 59 of the NCI-60 cell lines were genotyped to identify the presence of the Q141K allelic variant, previously shown to have diminished function compared with wild-type ABCG2 (Imai et al., 2002; Morisaki et al., 2005), as well as a SNP variant in the first intron of the gene recently found to correlate with chemotherapy toxicity in vivo (Rudin et al., 2008). An additional six variant sites were also genotyped (Table 1). For the Q141K SNP, 12 cell lines were found to contain a variant allele. Two lines were homozygous for this variant (LOX IMVI and A498). The other 10 lines were heterozygous, containing one Q141K variant allele (A549, COLO205, HCT116, SF295, MALME-3M, SK-OV-3, CAKI-1, HOP62, HOP92, and MDA-MB-231).

Twenty cell lines contained the intron one variant. Four cell lines were homozygous variant (SW620, OVCAR 5, BT549, and T47D), whereas 16 were heterozygous variant (MOLT4, HOP-92, HCC-2998, SF539, SNB19, SNB75, U251, SKMEL5, OVCAR3, OVCAR8, 786-0, RXF393, TK10, NCI ADR-RES, MDA-MB-231, and HS578T). For the other six variant sites, all 60 cell lines were homozygous wild type at each SNP site.

To determine whether these variants affected the correlation between gene expression and transporter function, the scatter plot analysis comparing expression with function was repeated after excluding those cell lines with the Q141K SNP or the intron 1 SNP. There was no clear pattern of improvement in correlation across these measures of gene expression and ABCG2 function (data not shown).

COMPARE Analysis. We next used the COMPARE program to probe the drug sensitivity database using the ABCG2 function profile. Because higher ABCG2 expression or function should lead to higher cellular resistance to compounds effluxed by the drug transporter, we hypothesized that our profile of ABCG2 levels across the 60 cell lines could be correlated with the cytotoxicity profiles of substrate drugs, as was observed for P-glycoprotein (Lee et al., 1997; Alvarez et al., 1998). The COMPARE correlation analysis produces a Pearson correlation coefficient (PCC) correlating ABCG2 levels with the sensitivities of the 60 cell lines to the compounds in the database.

In the COMPARE program, search parameters can be adjusted, including the requirement for a minimum number of cell lines that correlate with the probe and a minimum S.D. Through repeated testing and comparing the fingerprints of identified agents, we determined that optimized settings were: 30 cell lines and S.D. > 0.1. Because the majority of cell lines had modest and similar functional measures, we chose the lower setting of 30 cell lines (of 60) with which the ABCG2 fingerprint and a compound had to correlate to give a positive result. The S.D. of > 0.1 ensured that compounds had a significant variation across the 60 cell lines, and that a high PCC value showing correlation between the compound and ABCG2 function was not driven by a single or a few cell lines.

We probed the drug sensitivity database using the profile produced by the ABCG2 functional data to identify compounds with a high positive or negative correlation. The PCC is highly positively correlated with values approaching 1.0 and highly negatively correlated with values approaching −1.0. Values that range between −0.3 and +0.3 are thought to be not significantly correlated in the screen. Figure 2, right, shows the ABCG2 fingerprint generated by measuring FTC-inhibitable efflux in the cell lines. This fingerprint was used as the seed in the COMPARE algorithm.

From the NCI DTP web-accessible database, we obtained the cytoxicity profiles of 175 commonly used chemotherapy agents whose mechanisms are well understood, included as the NCI Anticancer Drug Screen's Standard Agents Database. This profile includes the GI_{50} of a compound for each of the 60 cell lines. Using COMPARE, we related the cytotoxicity profile for each standard agent to ABCG2 function in each cell line using the Pearson's correlation coefficient as well as the Spearman's rank correlation coefficient. There was no significant correlation between the ABCG2 functional fingerprint and the cytotoxicity profiles. As seen in Fig. 3, compounds known to be ABCG2 substrates such as mitoxantrone and topotecan had surprisingly lower than expected correlations of 0.082 and 0.011, respectively. The few exceptions to the generally low PCC values were a significant positive correlation with the agent pancratistatin (PCC = 0.447) and a negative correlation with ftorafur (PCC = −0.437). Reanalysis omitting cell lines known to express high

TABLE 1
ABCG2 genetic variants in the NCI-60 cell lines
Genotyping of *ABCG2* was performed as described under *Materials and Methods*.

Nucleotide Change	Amino Acid	Reference Sequence	Cell Lines	
			Heterozygote Variants	Homozygote Variants
	Intron 1	rs2622604	MOLT4, HOP-92, HCC2998, SF539, SNB19, SNB75, U251, SKMEL5, OVCAR3, OVCAR8, RXF393, TK10, NCI ADR-RES, MDA-MB-231, HS578T, 786−0	SW620, OVCAR 5, BT549, T47D
914C>A	Q141K	rs2231142	A549, COLO205, HCT116, SF295, MALME-3M, SK-OV-3, CAKI-1, HOP62, HOP92, MDA-MB-231	LOX IMVI, A498
862C>T	Y123Y	rs2231139	None	None
989C>G	Q166E	rs1061017	None	None
1057A>G	G188G	rs3116439	None	None
1116T>C	F208S	rs1061018	None	None
1235T>C	S248P	rs3116448	None	None
1493G>T	E334*	rs3201997	None	None

levels of P-gp or MRP1 did not improve PCCs (data not shown).

The ABCG2 functional fingerprint was then used as a probe in the larger collection of more than 100,000 synthetic agents that have been studied in the NCI 60. The compounds vary in terms of how well they are characterized, both molecularly and as potential anticancer agents. With this larger collection, only 187 compounds were found to have a positive correlation greater than 0.4 or a negative correlation less then −0.4. One compound (NSC651644) was found to have a PCC value above 0.6, four compounds had values above 0.5 (NSC722812, NSC686342, NSC625546, and NSC114609), and 66 compounds had correlations measuring between 0.4 and 0.5 (Table 2). A positive correlation in this setting could indicate that these compounds were ABCG2 substrates. The fingerprint of one compound with a positive PCC (0.456), NSC107392, is shown in Fig. 2, left.

Also found from this COMPARE analysis were compounds with negative correlations. Among the compounds found to have a high negative correlation with the ABCG2 functional fingerprint was NSC651424 (PCC = −0.739). Two additional compounds had correlations between −0.7 and −0.6, and another 23 compounds had PCC values between −0.6 and −0.5 (Table 2). Negative correlation in this context suggests that these agents are preferentially toxic to cells that express ABCG2 and could be considered potential ABCG2 "targeting" agents. This was found to be true in the case of P-gp, where some compounds with negative PCCs were found to be more toxic to cells overexpressing the transporter (Alvarez et al., 1995; Szakács et al., 2004).

Available compounds with high positive and low negative PCCs were obtained from the NCI DTP and subjected to further testing. One limitation at this step was that many compounds with high correlations were not available, including NSC651424 (PCC = −0.739), 693023 (−0.663), and 671546 (−0.626). A total of 27 compounds were obtained, 17

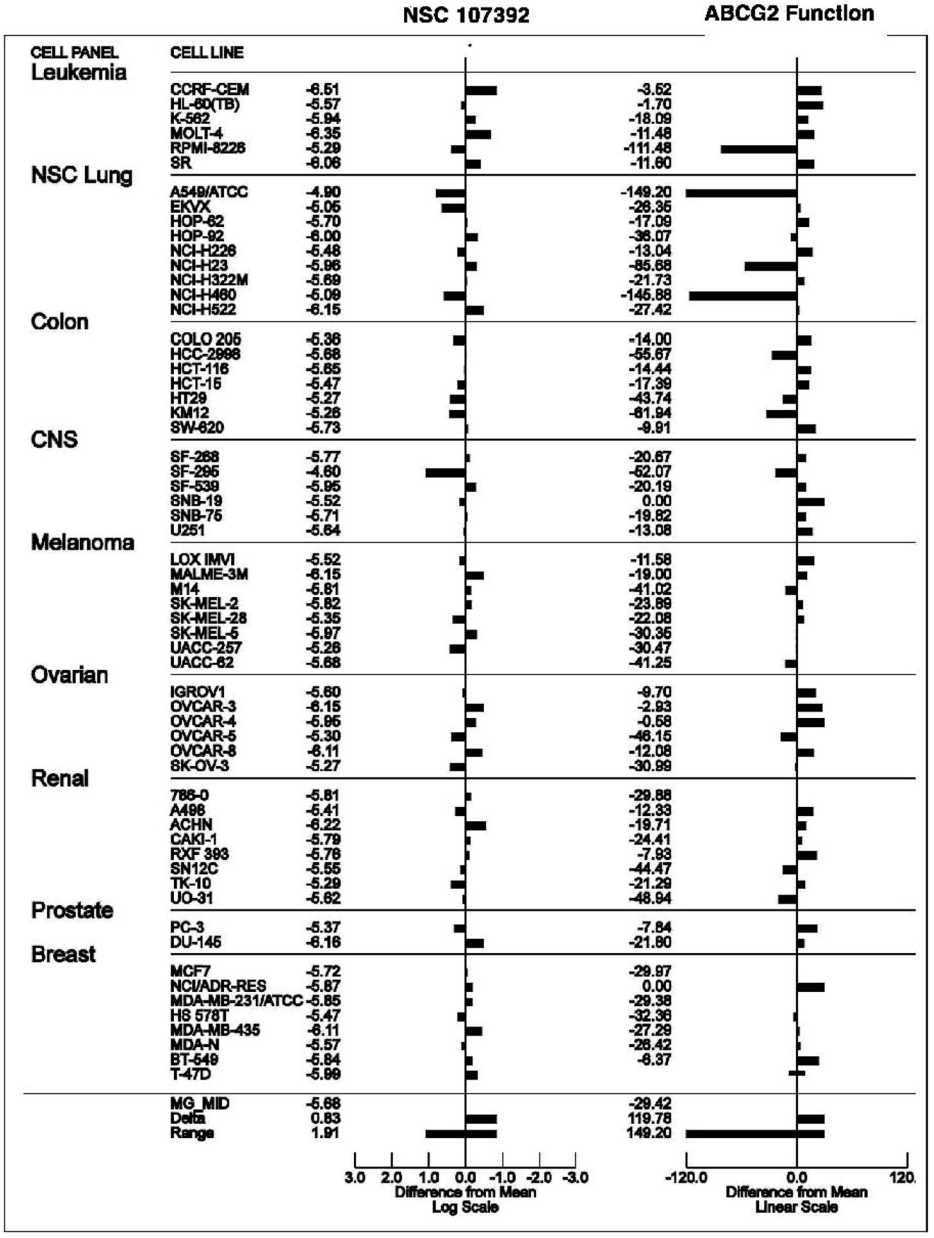

Fig. 2. Comparison of the mean graph (fingerprint) of ABCG2 function with the GI_{50} mean graph (fingerprint) of NSC107392 (PCC 0.456) across the NCI 60 cell lines. ABCG2 function: the vertical line represents the mean value across the 60 cell lines. The horizontal bars reflect the deviation from this mean for each cell line. Bars pointing to the left of the mean line are from cell lines with transporter function greater than the mean; bars pointing to the right are from cell lines with ABCG2 function less than the mean. NSC107392: in the mean graph (fingerprint) from this compound found to be a substrate for ABCG2, the vertical line represents the GI_{50} across all 60 cell lines. Bars pointing to the left represent cell lines requiring concentrations higher than the mean to kill 50% of the cells from time 0. Thus, cell lines with bars pointing to the left are more resistant to this ABCG2 substrate. Bars pointing to the right reflect cell lines that are more sensitive to the drug. It should be noted that the U251 and SNB-19 cell lines are reported to be from the same individual; the MDA-MB-435 cell line, listed as a breast cancer line, is suggested to be a melanoma; and the NCI/ADR-RES was determined to be derived from the OVCAR-8 cell line despite its being listed as a breast cancer by the drug screen.

with positive PCCs and 10 with negative PCCs (Table 3). We selected available compounds with a PCC ≥ 0.4 or ≤ -0.4 because these compounds generally had p values <0.005. Compounds with PCCs ≤ 0.35 tended to have p values greater than 0.05. In the case of some compounds, the PCC values shown represent the average PCC from several experiments in the drug screen.

Characterization of 27 Compounds Obtained by COMPARE Analysis. To determine whether any of the compounds were substrates of the ABCG2 transporter, 4-day cytotoxicity assays were performed with the 27 compounds on HEK293 cells transfected with empty pcDNA3.1 vector or vector containing DNA encoding the wild-type ABCG2 transporter. Relative resistance values were calculated by dividing the IC_{50} for each compound in the ABCG2-transfected cells by the IC_{50} for each compound in the empty vector-transfected cells. In cytotoxicity assays, ABCG2-expressing HEK cells are more resistant to a substrate than cells transfected with empty vector, resulting in a higher relative resistance value. ABCG2-expressing HEK cells were 19-fold resistant to topotecan compared with empty vector-transfected cells (Table 3). Substrates, identified as those compounds for which there was a greater than 2-fold relative resistance value, included 5 of the 27 compounds tested (NSC107392, NSC265473, NSC305458, NSC349156, and NSC382054) as listed in Table 3. ABCG2-transfected cells were particularly resistant to compounds NSC107392, NSC265473, and NSC349156, with relative resistance values greater than 37-fold, suggesting that these compounds are readily transported by ABCG2. During the course of our study, we discovered that NSC265473 and NSC305458 are actually the same compound, providing a serendipitous internal control.

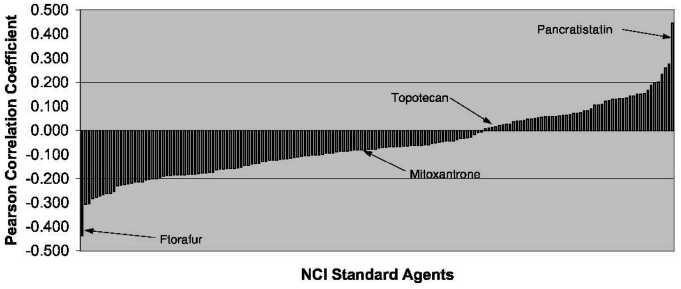

Fig. 3. Correlation between ABCG2 function and standard agents in NCI-ADS. PCC values are shown between the ABCG2 function fingerprint and the cytotoxicity profiles of 170 commonly used standard chemotherapy agents.

We next sought to determine whether resistance to the test compounds could be conferred by the lower endogenous levels of ABCG2 found in the unselected drug screen cell lines. Four-day cytotoxicity assays were performed on A549, NCI-H460, and HT29 cells with SN-38, topotecan, NSC107392, or NSC265473 in the presence or absence of 5 μM FTC, an ABCG2 inhibitor. Dose-modifying factors, representing sensitization as a result of incubation in the presence of FTC, were then calculated by dividing IC_{50} values obtained in the absence of FTC by IC_{50} values obtained in the absence of

TABLE 2

Compounds identified by the COMPARE algorithm as correlating with ABCG2 function

Compounds whose cytotoxicity profile or "fingerprint" across the NCI-60 cell lines have the highest positive and negative correlations with the ABCG2 functional results are listed, including the value of the Pearson correlation coefficient. Full chemical names for all compounds may be located at http://pubchem.ncbi.nlm.nih.gov.

Potential Substrates				Potential Targeting Agents			
Rank	Compound	PCC	Name	Rank	Compound	PCC	Name
1	NSC651644	0.635		1	NSC651424	−0.739	
2	NSC722812	0.592		2	NSC723121	−0.616	
3	NSC686342	0.516		3	NSC716430	−0.611	
4	NSC625546	0.506	Cordigone	4	NSC674449	−0.587	
5	NSC114609	0.503		5	NSC679209	−0.572	
6	NSC696102	0.492		6	NSC668352	−0.571	
7	NSC736740	0.489		7	NSC624663	−0.570	Betulitrin
8	NSC626879	0.479		8	NSC697538	−0.568	
9	NSC668355	0.478		9	NSC141539	−0.562	
10	NSC636795	0.477		10	NSC672223	−0.551	
11	NSC717452	0.476		11	NSC702426	−0.550	
12	NSC665697	0.476		12	NSC652295	−0.545	
13	NSC622161	0.471		13	NSC679280	−0.540	
14	NSC103054	0.471	2,4-Dibromoestradiol	14	NSC664904	−0.538	
15	NSC305458	0.468		15	NSC661963	−0.538	
16	NSC715406	0.464		16	NSC668351	−0.530	
17	NSC623636	0.464		17	NSC601348	−0.529	
18	NSC620515	0.463		18	NSC660031	−0.522	
19	NSC652903	0.462	Saframycin	19	NSC691529	−0.520	
20	NSC630986	0.458		20	NSC691209	−0.519	
21	NSC621888	0.458		26	NSC153330	−0.502	
22	NSC655897	0.457		29	NSC45384	−0.498	ME-Streptonigrin
23	NSC107392	0.456	5-HP	30	NSC58368	−0.497	Fumagillin
24	NSC174137	0.456		42	NSC24048	−0.472	
25	NSC175150	0.455	Rosamicin	47	NSC69574	−0.466	
26	NSC349156	0.447	Pancratistatin	48	NSC382054	−0.465	
27	NSC691417	0.440		70	NSC600391	−0.439	
28	NSC620303	0.438		81	NSC148958	−0.431	Tegafur
29	NSC691782	0.438		104	NSC608001	−0.409	
32	NSC313981	0.436					
36	NSC297093	0.430					
72	NSC274557	0.400					
74	NSC265473	0.399					

FTC. In general, dose-modifying factor values were lower for SN-38 (range, 1–2.7) and topotecan (range, 1–2.6) than for the two novel substrates NSC107392 (range, 1.4–3.0) and NSC265473 (range, 2.1–6.5). However, these differences did not achieve statistical significance.

The negatively correlating compounds were expected to be more toxic to cells expressing ABCG2. However, only 2 of the 27 tested compounds (NSC103054 and NSC174939) showed evidence of greater cytotoxicity in ABCG2-expressing cells with relative resistance values less than 0.5. One of two compounds (NSC103054) that was more toxic to *ABCG2*-transfected cells had a positive PCC (PCC = 0.471). Four of the 10 compounds with negative PCCs were not toxic to either of the two transfected cell lines at concentrations of up to 100 μM.

We also performed cytotoxicity assays with a subset of the 27 compounds on parental NCI-H460 and ABCG2-overexpressing NCI-H460 MX20 cells. As seen in supplemental Table 2, similar relative resistance values were observed and did not differ from those for the transfected cells (Table 3) by more than 3-fold except in the case of NSC349156, where the transfected cells had relative resistance values that were more than 4-fold higher than for the NCI-H460 and NCI-H460 MX20 cells.

Next, we sought to determine whether these 27 compounds were able to interact with ABCG2 by testing their ability to inhibit photo-cross-linking of ABCG2 with [^{125}I]IAAP as described above. This method has been used to identify compounds that bind to ABCG2 at the same site as prazosin, a known ABCG2 substrate, by measuring their ability to prevent binding of the radiolabeled, photo-cross-linkable analog of prazosin, [^{125}I]IAAP, to ABCG2. An example of the gel results from this assay is shown in Fig. 4A. Results for each compound are listed in Table 3, with results reported as the percentage inhibition of ABCG2 labeling (i.e., 80% inhibited for FTC). Eight of the 17 compounds with a positive PCC inhibited IAAP binding by more than 50% (NSC651644, NSC103054, NSC623636, NSC620515, NSC691417, NSC620303, NSC297093, and NSC265473). In addition, 5 of 10 compounds with a negative PCC inhibited IAAP binding by more than 50% (NSC153330, NSC45384, NSC24048, NSC382054, and NSC608001).

As another measure of interaction with ABCG2, positively or negatively correlating agents were tested for their ability to inhibit ABCG2 function by determining the effect of 10 μM concentrations of the compounds on the efflux of PhA from wild-type *ABCG2*-transfected cells. *ABCG2*-transfected cells were incubated in PhA in the presence (dashed line) or absence (solid line) of 10 μM concentrations of each compound, and the -fold-increase in PhA fluorescence in the dashed histogram compared with the solid histogram was calculated. Results obtained with five of the tested compounds and the positive control FTC are given in Fig. 4B; results for all compounds are summarized in Table 3. Three of 10 compounds tested with a negative PCC were found to increase PhA fluorescence by 2-fold or more (NSC24048, NSC45384,

TABLE 3

Summary of test data for compounds identified as correlating with ABCG2 function as measured by PhA transport

Compounds marked nontoxic were not cytotoxic at concentrations up to 100 μM. ABCG2-transfected cells were 19-fold resistant to topotecan compared to empty vector-transfected cells. Where *p* values were not determined, the PCC represents an average of several PCCs for different experiments with a given compound in the drug screen. Relative resistance was calculated by dividing the IC_{50} of each compound for the ABCG2-transfected cell line by the IC_{50} value for the empty vector transfected cell line. Values given are means from at least two independent experiments. Percentage inhibition of IAAP labeling of ABCG2 was determined after normalizing the value of all bands to the value obtained for IAAP incorporation into the untreated band, which was set to 100%. Values are means from two independent experiments. Increase in PhA fluorescence was determined by dividing the intracellular fluorescence of PhA in the presence of 10 μM inhibitor by the PhA fluorescence in the absence of inhibitor. Values are means from at least two independent experiments. Full chemical names for all compounds may be located at http://pubchem.ncbi.nlm.nih.gov.

Compound	Pearson Correlation Coefficient	*P* Value	Relative Resistance	IAAP Binding Inhibited	Pheophorbide Fluorescence
				%	*-fold increase*
NSC636795	0.48	6.1×10^{-4}	0.94	15	2.4
NSC103054	0.47	N.D.	0.33	81	3.1
NSC305458[a]	0.47	9.1×10^{-4}	38	45	0.99
NSC623636	0.46	3.4×10^{-3}	1	99	1.1
NSC620515	0.46	N.D.	1.1	70	1.3
NSC621888	0.46	N.D.	0.86	0	1.1
NSC107392	0.46	N.D.	47	30	0.97
NSC174137	0.46	3.2×10^{-4}	0.96	43	0.9
NSC175150	0.46	3.8×10^{-4}	0.8	15	1.2
NSC349156	0.45	3.9×10^{-4}	37	17	0.92
NSC691417	0.44	7.8×10^{-4}	0.92	62	2
NSC620303	0.44	1.8×10^{-3}	0.86	65	1
NSC691782	0.44	9.1×10^{-4}	1.2	25	1.1
NSC313981	0.44	1.9×10^{-3}	1.2	30	1.3
NSC297093	0.43	1.8×10^{-3}	1.5	65	1
NSC274557	0.40	4.8×10^{-3}	1.2	22	1.8
NSC265473[a]	0.40	5.4×10^{-3}	98	79	1
NSC608001	−0.41	N.D.	0.7	80	2
NSC148958	−0.43	4.8×10^{-4}	Nontoxic	25	0.94
NSC600391[b]	−0.44	5.0×10^{-4}	Nontoxic	0	1.2
NSC174939	−0.45	N.D.	0.4	30	1.2
NSC382054	−0.46	N.D.	2.4	60	1.2
NSC69574	−0.47	6.5×10^{-4}	0.9	27	1.3
NSC24048	−0.47	2.7×10^{-4}	Nontoxic	78	15
NSC58368	−0.50	3.7×10^{-4}	Nontoxic	30	1
NSC45384	−0.50	3.7×10^{-4}	1.9	85	12
NSC153330	−0.50	6.1×10^{-5}	0.92	73	1.8

N.D., not determined.
[a] The IC_{50} value for the ABCG2 expressing line was not achieved; thus, the actual relative resistance value is likely higher.
[b] The molecular weight of this compound is not known. The compound was nontoxic up to a concentration of 100 μg/ml.

and NSC608001). In addition, three of 17 compounds with a positive correlation were found to increase intracellular PhA fluorescence by 2-fold or greater (NSC636795, NSC103054, and NSC691417). Structures for selected test compounds are provided in Fig. 4C. Structures of other compounds can be obtained from http://dtp.nci.nih.gov.

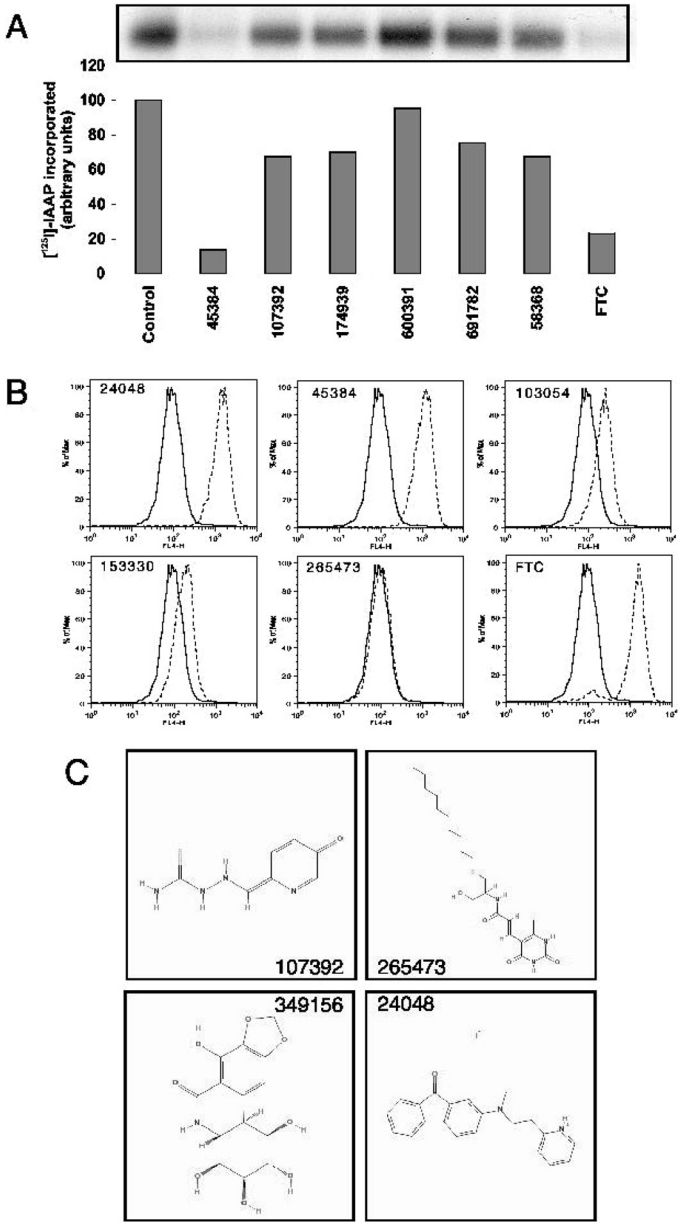

Fig. 4. Interaction of compounds identified by the NCI-ADS with ABCG2. A, crude membranes isolated from MCF-7 FLV1000 cells were incubated with 20 μM concentrations of the compounds in the presence of [^{125}I]IAAP followed by UV crosslinking and immunoprecipitation of ABCG2 as outlined under *Materials and Methods*. An autoradiogram from a representative experiment is shown. Membranes were also incubated with 20 μM FTC as a positive control for inhibition of labeling of ABCG2 by [^{125}I]IAAP. B, inhibition of PhA efflux was measured in wild-type *ABCG2*-transfected cells by incubating cells in 1 μM PhA in the presence or absence of 10 μM concentrations of the compounds identified by COMPARE analysis for 30 min. Subsequently, cells were washed and allowed to incubate in PhA-free medium for 1 h continuing with (dashed line) or without (solid line) 10 μM concentrations of the compounds. Histograms representative of one of at least two experiments are shown. Cells incubated in 10 μM FTC serve as a positive control for inhibition of ABCG2-mediated PhA transport. C, chemical structures of selected ABCG2-interacting compounds.

Of the six compounds that were able to increase PhA fluorescence in the *ABCG2*-transfected cells by at least 2-fold, four compounds that were nontoxic at concentrations shown to inhibit ABCG2 function were further tested in combination studies with topotecan, a cytotoxic agent known to be a ABCG2 substrate. Cytotoxicity asssays were performed on *ABCG2*-transfected and empty vector-transfected HEK293 cells with topotecan at various concentrations, along with nontoxic concentrations of the compounds NSC608001 (10 μM, PCC = -0.409), NSC636795 (10 μM, PCC = 0.477), NSC153330 (25 μM, PCC = -0.502), and NSC24048 (10 μM, PCC = -0.472). In all cases, the concentrations chosen resulted in less than 10% cell death of both the empty vector-transfected and the ABCG2-transfected cells. Although NSC608001, NSC636795, and NSC153330 partially reversed ABCG2-mediated topotecan resistance in *ABCG2*-transfected cells, NSC24048 completely reversed ABCG2-mediated resistance (Table 4).

Discussion

The NCI-ADS has proven to be a powerful basic and translational research tool to identify new anticancer agents, characterize cytotoxic drug activity, and investigate mechanisms of cancer drug resistance. We and others have previously used this research tool and database to identify agents that targeted the epidermal growth factor receptor (Wosikowski et al., 1997; Liu et al., 2007), the erbB2 pathway (Wosikowski et al., 1997) and Chk2 (Jobson et al., 2007), as well as agents that were substrates of the drug transporters P-gp (Lee et al., 1994) and MRP1 (Alvarez et al., 1998).

In the current study, we characterized the function of the ABCG2 efflux transporter across the 60 cell lines of the NCI-ADS. Our hypothesis was that a measure of transporter function might be more sensitive than gene expression to identify compounds that interact with ABCG2, a strategy that in the past has been used successfully to identify substrates for other ABC transporters. Using *ABCG2* gene expression measured by real-time reverse transcription-polymerase chain reaction as a fingerprint probe, Szakács et al. (2004) did not identify transporter substrates within the drug screen. As shown here, using transporter function as a fingerprint to probe the screen did identify novel substrates and inhibitors of this critical drug transporter.

Although we did identify potential substrates and targeting agents using transporter function as the seed probe entered into the COMPARE program, in general we were unable to identify as many compounds as were identified previously when P-glycoprotein function was used as the seed (Lee et al., 1994). Using PhA efflux as a measure of ABCG2 function, only one compound was found to have a PCC value above 0.6, only four compounds had values above 0.5, and 66 compounds measured between 0.4 and 0.5. Although these values are low compared with correlations obtained using P-gp function as the seed, where PCC values for the top 20 compounds ranged from 0.816 to 0.976, they are in the same range as that observed for several molecular targets, including MRP1 (Alvarez et al., 1998) and epidermal growth factor receptor (Wosikowski et al., 1997). Known substrates of ABCG2 that are found in the drug screen, such as mitoxantrone, topotecan, and SN-38 (the active metabolite of irinotecan), were surprisingly not found to significantly cor-

relate with ABCG2 function, leading us to the conclusion that the screen cannot be used as a predictive tool to determine whether or not a compound is a substrate. We were, however, able to identify the novel ABCG2 substrates NSC107392, NSC265473, and NSC349156 (pancratistatin). Pancratistatin is under investigation for its antitumor properties (McLachlan et al., 2005). It is noteworthy that a previously reported P-gp targeting agent, a thiosemicarbazone, was also shown to be an ABCG2 substrate (Wu et al., 2007).

There are several possible explanations for the somewhat low PCC values obtained and why known substrates were not identified. First, the range of ABCG2 function across the cell lines in the screen is not as broad as that for P-gp. When values are widely separated across the 60 cell lines, stronger correlations are possible. Few cell lines have relatively high function of ABCG2 as measured by PhA efflux (A549, NCI-H460, RPMI 8226, and NCI-H23). This compares to 12 cell lines found to have high P-gp function in our previous work, some at levels found in drug-selected cell lines (Lee et al., 1994). The generally low levels of ABCG2 expression in the cell lines of the drug screen would also serve to reduce correlations for compounds that are relatively poor substrates of ABCG2 such as flavopiridol, because high levels of ABCG2 expression are needed to confer resistance to such compounds. Ultimately, including more cell lines with higher levels of ABCG2 in the drug screen might allow for identification of known ABCG2 substrates. Second, the contribution of the drug target might obscure the contribution of ABCG2 to resistance. Cancer cells that express high levels of a drug target, such as topoisomerase, may be exquisitely sensitive to drugs targeting the protein such that ABCG2 is not able to keep intracellular drug concentrations low enough to confer resistance. Likewise, ABCG2 substrates such as imatinib would not be identified as substrates by the drug screen unless cell lines expressed the BCR-ABL fusion protein or an activating KIT mutation. Third, expression of other transporters, such as P-gp or MRP1, may obscure the contribution of ABCG2 to drug resistance in a given cell line. Some in vitro studies support such a hypothesis, because, for example, topotecan uptake into the brain was not observed to be increased in Abcg2-deficient mice until the Mdr1/2 genes were also deleted (de Vries et al., 2007). Another possibility is that SNPs may directly affect ABCG2 function. A polymorphism that impairs function could readily explain the failure of gene expression data to correlate with cytotoxicity data. However, a functional measure should account for any influence of polymorphic forms of ABCG2 as we presently understand them. Furthermore, our analyses on the correlation between function and expression, as well as the correlation between compound cytotoxicity and function, did not improve when genetic variant cell lines were excluded. Finally, ABCG2 localization may have an impact on drug resistance. Intracellular expression of ABCG2 has been observed in some tissue samples, and it is unknown whether intracellular ABCG2 can mediate drug resistance. If so, measuring PhA efflux might underestimate the contribution of ABCG2 to drug resistance in some cell lines. If this were true, measurement of ABCG2 by immunoblot should improve correlations; again, however, levels of ABCG2 remain generally low in the 60 cell lines of the screen. Nonetheless, measurement of ABCG2 function did allow us to identify novel compounds that interact with ABCG2.

In this analysis we also looked for correlations with PCC values that were negative to identify compounds that might target cells that express high levels of ABCG2. Of the 27 compounds that we examined, only two were slightly more toxic (2.5- and 3-fold) in ABCG2-transfected cells versus empty vector transfected cells. Because the COMPARE program was able to identify compounds with negative PCCs that selectively target cells that overexpress P-gp (Szakács et al., 2004), it is possible that similar compounds that are selectively toxic to ABCG2-overexpressing cells could be identified.

ABCG2-targeting compounds may be of interest for a novel clinical approach—that of targeting putative cancer stem cells. Normal hematopoietic stem cells as well as cancer stem cells are identified by flow cytometry in a distinct population of cells that stain dimly with the fluorescent dye Hoechst 33342. This so-called "side population" is due to ABCG2-mediated Hoechst efflux and is enriched in tumorigenic or potential cancer stem cells (Zhou et al., 2001). Whether true cancer stem cells exist or not is still hotly debated, but it does appear that cells in this side population could have intrinsic chemotherapy resistance as a result of ABCG2-mediated drug efflux. Therefore, a potential therapeutic modality would be to use compounds that are selectively toxic to this subset of cells.

Of the 27 compounds tested, six were found to inhibit ABCG2-mediated PhA transport in ABCG2-transfected cells at 10 μM. In particular, NSC24048, NSC153330, NSC608001, and NSC636795 were sufficiently nontoxic that combination cytotoxicity assays could be performed. It is noteworthy that these included compounds with both positive and negative PCCs. NSC24048 was the most potent compound tested and completely reversed ABCG2-mediated topotecan resistance in the ABCG2-transfected cells at a concentration of 10 μM. The identification of potential inhibitors of the ABCG2 drug transporter may prove to be clinically useful given the central role that ABCG2 plays in the blood-brain barrier and in mediating oral absorption of drugs. ABCG2 inhibitors may provide increased delivery of chemotherapy agents into sanctuary sites such as the brain

TABLE 4

Reversal of ABCG2-mediated topotecan resistance by compounds NSC24048, NSC153330, NSC608001, and NSC636795

Relative resistance (RR) values were obtained by dividing the IC_{50} values in R-2 cells for topotecan in the absence or presence of the desired compound by the IC_{50} value for the pcDNA cells without compounds added. Values are mean ± standard deviation. At least three independent experiments were performed.

Drug	pcDNA	R-2	RR
Topotecan	0.021 ± 0.008	0.39 ± 0.06	19
Topotecan + 10 μM NSC24048	0.026 ± 0.01	0.019 ± 0.005	0.73
Topotecan + 25 μM NSC153330	0.018 ± 0.01	0.087 ± 0.03	4.8
Topotecan + 10 μM NSC608001	0.015 ± 0.001	0.10 ± 0.01	6.7
Topotecan + 10 μM NSC636795	0.017 ± 0.004	0.083 ± 0.02	4.9

to treat primary or metastatic disease (Breedveld et al., 2005).

In conclusion, measurements of ABCG2 transporter function were more successful than gene expression when used as a probe to identify drugs that interact with this transporter. We identified novel cytotoxic agents that are substrates of ABCG2, as well as some drugs that functioned as transport inhibitors. Further investigations and preclinical evaluation of these substrates and inhibitors are warranted and are ongoing.

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