

# New inhibitors of ABCG2 identified by high-throughput screening

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## Abstract

In order to identify novel inhibitors of the ATP-binding cassette transporter, ABCG2, a high-throughput assay measuring the accumulation of the ABCG2 substrate pheophorbide a in ABCG2-overexpressing NCI-H460 MX20 cells was used to screen libraries of compounds. Out of a library of 7,325 natural products and synthetic compounds from the National Cancer Institute/Developmental Therapeutics Program collection, 18 were found to inhibit ABCG2 at 10  $\mu\text{mol/L}$ . After eliminating flavonoids and compounds of limited availability from the 18 original compounds, 10 of the 11 remaining compounds reversed mitoxantrone resistance in NCI-H460/MX20 cells and prevented ABCG2-mediated BODIPY-prazosin transport in ABCG2-transfected HEK293 cells, confirming an interaction with ABCG2. Based on the activity profiles and the availability of materials, five inhibitors were examined for their ability to compete with [<sup>125</sup>I]iodoarylazidoprazosin labeling of ABCG2, increase binding of the anti-ABCG2 antibody 5D3, and prevent P-glycoprotein or multidrug resistance protein 1-mediated transport. At a concentration of 20  $\mu\text{mol/L}$ , all of the compounds reduced iodoarylazidoprazosin labeling by 50% to 80% compared

with controls. All five compounds also increased 5D3 labeling of ABCG2, indicating that these compounds are inhibitors but not substrates of ABCG2. None of the compounds affected P-glycoprotein-mediated rhodamine 123 transport, whereas three affected multidrug resistance protein-1-mediated calcein transport at 25  $\mu\text{mol/L}$ , suggesting that the compounds are relatively specific for ABCG2. These five novel inhibitors of ABCG2 activity may provide a basis for further investigation of ABCG2 function and its relevance in multidrug resistance. [Mol Cancer Ther 2007;6(12):3271–8]

## Introduction

The acquisition of multidrug resistance has long been recognized as a major obstacle to successful cancer chemotherapy. The multidrug resistance transporter ABCG2 (or breast cancer resistance protein 1, BCRP1), a member of the ATP-binding cassette (ABC) family of membrane transport proteins, is believed to form a part of the maternal-fetal barrier, the blood-brain barrier, and is known to limit the oral absorption of some drugs (1). The normal physiologic function(s) of ABCG2 may be related to the transport of a variety of natural substances to prevent intracellular accumulation of toxic compounds. ABCG2 is also an important mediator of resistance to a variety of anticancer drugs including mitoxantrone, topotecan, irinotecan, flavopiridol, and methotrexate (2–5). Thus, inhibitors of ABCG2 activity could have important oncologic and pharmacologic applications. In contrast to related ABC transporters such as P-glycoprotein (Pgp), few if any clinically useful inhibitors of ABCG2 activity have been developed. In addition to obvious potential therapeutic relevance, the development of new specific modulators of ABCG2 will have considerable utility in advancing the understanding of ABCG2 function.

ABCG2 expression has been reported in a wide variety of untreated human solid tumors and its expression has been implicated in drug resistance in acute myeloid leukemia. Diestra and colleagues were among the first to study a large number of solid tumor samples, finding relatively high expression in tumors of the lung, endometrium, and digestive tract as well as melanoma (6). ABCG2 expression in non-small cell lung cancer tumors was reported to be predictive of a lower response rate in patients receiving platinum-based therapy, despite the fact that platinum compounds are not substrates of ABCG2 (7). Several studies have reported that ABCG2 expression affects the response to chemotherapy or affects progression-free survival (8–10). Gene expression profiling of pretreatment samples from 170 patients with acute myeloid leukemia revealed a cluster group characterized by high ABC transporter expression and highly resistant disease (11). ABCG2 is also highly expressed in normal and in putative

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cancer stem cells (12). Inhibitors may therefore increase initial response to chemotherapy or may be useful to gain increased accumulation of molecularly targeted agents to various cancer populations.

Inhibition of ABCG2 is also being pursued to increase oral bioavailability and brain penetration of ABCG2 substrates. Previous studies have shown that coadministration of the ABCG2 inhibitor elacridar with topotecan enhanced oral bioavailability in mice (13), whereas coadministration of gefitinib and irinotecan enhanced oral bioavailability and antitumor activity in mice (14). Additionally, the inhibition of transporters expressed at the blood-brain barrier, such as Pgp and ABCG2, has been shown to increase brain penetration of gefitinib (15) and topotecan (16), highlighting the need for potent inhibitors of ABCG2.

The search for ABCG2 inhibitors dates from the discovery of the ability of fumitremorgin C (FTC) to reverse non-Pgp, non-multidrug resistance protein (MRP) multidrug resistance in a resistant cell line later shown to overexpress ABCG2 (17).

A high-throughput inhibitor screening assay based on the accumulation of a fluorescent ABCG2 substrate, pheophorbide a (PhA), has recently been developed (18). Libraries of synthetic and natural products comprising 7,325 compounds were obtained from the National Cancer Institute (NCI), Developmental Therapeutics Program (DTP) and screened using this assay. The DTP repository of compounds has proven to be a rich source of both synthetic compounds and natural products as molecularly targeted reagents (19, 20). When applied in the ABCG2 screen, several compounds were identified as novel inhibitors of ABCG2 activity. Activities were confirmed by multiple additional ABCG2 assays. These included sensitization of ABCG2-overexpressing cells to killing by mitoxantrone and activity against ABCG2 in transfected cells. Five of the active compounds were further characterized. Because ABCG2 has overlapping substrate specificity with MRP1 and Pgp and because Pgp, MRP1, and ABCG2 are the major contributors to multidrug resistance in most cancer cells in culture (4), the selected compounds were tested for their ability to affect MRP1 and Pgp activities.

## Materials and Methods

### Materials

PhA was obtained from Frontier Scientific. FTC, libraries of pure natural products, and synthetic molecules (structural diversity and mechanistic diversity sets) and individual compounds were obtained from the Drug Synthesis and Chemistry Branch, DTP, Division of Cancer Treatment and Diagnostics, NCI (Bethesda, MD). Cell culture media were from Invitrogen, fetal bovine serum was from Hyclone, and PBS was from Quality Biological.

### Cell Culture

NCI-H460 human lung non-small cell carcinoma cells (National Cancer Institute) were selected for overexpression of ABCG2 by maintenance in RPMI 1640/10% fetal bovine serum supplemented with 20 nmol/L of mitoxantrone (21).

After the removal of mitoxantrone, cells were further grown in the same medium without mitoxantrone for 5 to 30 days. These cells were designated NCI-H460/MX20. Parental cells (low ABCG2 expression; ref. 21) were maintained in the same medium without mitoxantrone. ABCG2-transfected or MDR1-transfected (i.e., Pgp-expressing) HEK293 cells were maintained in 2 mg/mL of G418 as previously described (22). MRP1-transfected HEK293 cells were maintained in 5  $\mu$ mol/L of etoposide. MCF-7 FLV1000 cells were maintained in Richter's medium with 10% FCS and penicillin/streptomycin with 1,000 nmol/L of flavopiridol (23).

### Screening Assay for ABCG2 Inhibitors

The accumulation of PhA, a fluorescent ABCG2 substrate (24, 25), formed the basis of the assay for inhibitors of ABCG2 activity (18). Briefly, NCI-H460/MX20 cells were transferred to black-walled, clear-bottomed 384-well polylysine-coated assay plates (Corning) and allowed to attach for several hours. PhA (1  $\mu$ mol/L final concentration) was added, immediately followed by compounds or vehicle (DMSO/PBS) control and incubated for an additional 18 h. After removal of medium and washing with PBS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , fluorescence intensity was read on a Tecan Safire fluorescence plate reader in bottom read mode (395 nm excitation, 670 nm emission). Each plate had control wells containing 10  $\mu$ mol/L (final concentration) of FTC. Data were normalized to FTC and reported as the percentage of FTC fluorescence.

### Mitoxantrone Sensitization

The ability of compounds to sensitize NCI-H460/MX20 cells to killing by mitoxantrone was assessed as described (18). ABCG2-overexpressing cells or parental cells were treated with mitoxantrone in the presence or absence of 10  $\mu$ mol/L of compound (or 1  $\mu$ mol/L FTC) and cell numbers assessed after 2 days by an XTT assay (26). Final DMSO concentration was 0.2% (v/v).

### Flow Cytometry

Compounds identified in the screen were confirmed for their ability to inhibit ABCG2-mediated transport using BODIPY-prazosin as a substrate (22). Five of these were additionally tested for their ability to inhibit Pgp-mediated rhodamine 123 efflux and MRP1-mediated calcein efflux as previously described (22, 27). Briefly, transfected HEK293 cells expressing ABCG2, Pgp, or MRP1 were trypsinized and incubated in complete medium (phenol red-free Richter's medium with 10% FCS and penicillin/streptomycin) containing 200 nmol/L of BODIPY-prazosin, 0.5  $\mu$ g/mL of rhodamine 123, or 200 nmol/L of calcein AM, respectively, in the presence or absence of the desired concentration of inhibitor for 30 min at 37°C. The positive controls for inhibition of ABC transporters were 10  $\mu$ mol/L of FTC for ABCG2, 3  $\mu$ g/mL of valsopodar for Pgp, and 25  $\mu$ mol/L of MK-571 for MRP1. Cells were then washed and incubated in substrate-free medium continuing with or without inhibitor for 1 h.

The 5D3 shift assay was done as described by Ozvegy-Laczka and colleagues with minor modifications (28). ABCG2-transfected HEK293 cells were trypsinized and incubated with 5D3 antibody (1:3,500; eBioscience) for

2 h in the presence or absence of 20  $\mu\text{mol/L}$  of each of the compounds or 20  $\mu\text{mol/L}$  of FTC as a positive control. Cells were subsequently washed and then incubated with APC-labeled goat anti-mouse secondary antibody (1:35) for 30 min after which the cells were washed and analyzed.

Intracellular fluorescence of BODIPY-prazosin, rhodamine 123, or calcein fluorescence was detected with a FACSort flow cytometer equipped with a 488 nm argon laser and 530 nm bandpass filter. APC fluorescence was measured with a 635 nm read diode laser and 561 nm longpass filter. At least 10,000 events were collected. Dead cells were eliminated based on propidium iodide exclusion.

#### Photoaffinity Labeling of ABCG2 with [ $^{125}\text{I}$ ]iodoarylazidoprazosin

ABCG2 expressed in MCF-7 FLV1000 cells was photo-labeled with [ $^{125}\text{I}$ ]iodoarylazidoprazosin (IAAP) as described previously (29). Briefly, crude membranes (1 mg protein/mL) of MCF-7 FLV1000 cells were incubated with 20  $\mu\text{mol/L}$  of the indicated compound for 10 min at room temperature in 50 mmol/L of Tris-HCl (pH 7.5). Three to 6 nmol/L of [ $^{125}\text{I}$ ]IAAP (2,200 Ci/mmol; Perkin-Elmer Life Sciences) was added and the samples were incubated for an additional 5 min under subdued light. The samples were then exposed to UV light (365 nm) for 10 min and the labeled ABCG2 was immunoprecipitated using BXP-21 antibody. The radioactivity incorporated into the ABCG2 band was quantified using the STORM 860 PhosphorImager system (Molecular Dynamics) and ImageQuaNT software (Molecular Dynamics).

#### Data Analysis

Apparent  $\text{IC}_{50}$  values were calculated from dose-response data using SigmaPlot (SPSS, Inc.) four-variable logistic nonlinear regression analysis. Unless otherwise noted, all data are presented as average  $\pm$  SE.

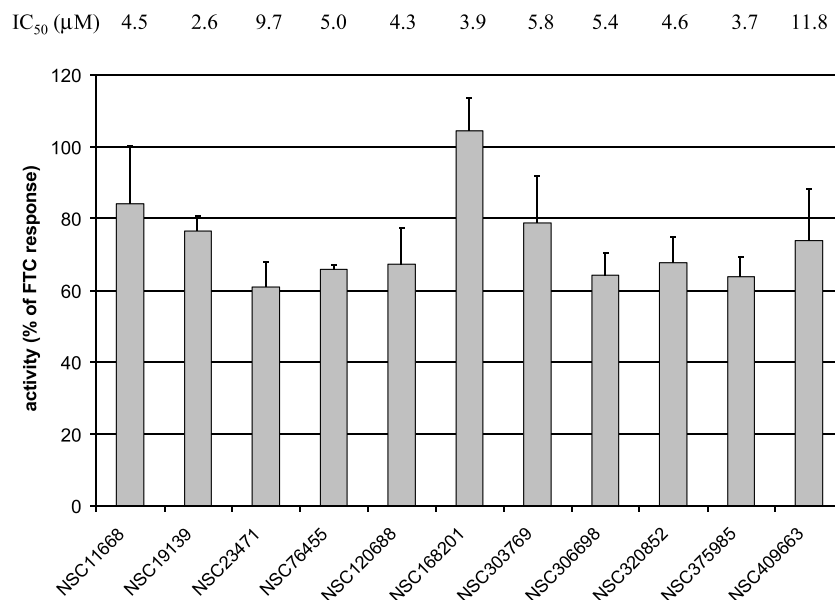
## Results

### Screening

A total of 7,325 pure natural products and synthetic compounds comprised the NCI-DTP compound libraries screened using the PhA assay. Out of these libraries, 18 compounds were identified and confirmed as "hits." Compounds identified as hits in screening (i.e.,  $\geq 50\%$  of FTC response) were reassayed in quadruplicate and results analyzed by calculation of confidence intervals. A "confirmed hit" was defined as a compound for which reassay showed  $\geq 50\%$  of FTC response at a 95% confidence interval (18). After the elimination of flavonoids (a well-characterized class of ABCG2 inhibitors) and compounds unavailable for resupply, 11 compounds were further characterized. Figure 1 summarizes the activities of these compounds in the PhA accumulation assay. The compounds had activities ranging from 60% to 105% of the activity of FTC when evaluated at 10  $\mu\text{mol/L}$ . In this assay,  $\text{IC}_{50}$  for FTC was 0.8  $\mu\text{mol/L}$  (18).

### Mitoxantrone Sensitization (ABCG2-Overexpressing Cells)

In order to confirm the functional relevance of the measured pheophorbide activities, each compound was tested for its ability to restore mitoxantrone sensitivity to cells overexpressing ABCG2 (Fig. 2). Unselected NCI-H460 cells were sensitive to killing by mitoxantrone. After 2 days in the presence of 30  $\mu\text{mol/L}$  of mitoxantrone, cell numbers were  $21.6 \pm 1.6\%$  (SD) of control (vehicle). None of the compounds tested were significantly cytotoxic against parental cells (data not shown). Cells selected for ABCG2 overexpression (NCI-H460/MX20) were significantly more resistant to mitoxantrone (see "PBS" column in Fig. 2). After mitoxantrone treatment, NCI-H460/MX20 cell number was  $56.1 \pm 2.1\%$  (SD) of controls. In the presence of 1  $\mu\text{mol/L}$  of FTC, this



**Figure 1.** Activity of compounds in the PhA screening assay. The compounds listed were assayed for their ability to cause ABCG2-overexpressing cells (NCI-H460/MX20 cells) to accumulate PhA. Each compound was tested at 10  $\mu\text{mol/L}$  (final concentration—bars) and, after resupply, in a dose-response format. Activity for each compound was normalized to the response of 10  $\mu\text{mol/L}$  FTC control wells on the same plate. Columns, mean; bars, SE ( $n = 7-9$ ).  $\text{IC}_{50}$  values are averages of duplicate determinations for each dose.

was further reduced to  $24.5 \pm 1.5\%$  (SD). Similar effects were seen with all of the tested compounds (at  $10 \mu\text{mol/L}$ ), except for NSC23471, which did not significantly sensitize the cells to mitoxantrone. None of the compounds alone caused significant cell killing in the NCI-H460/MX20 subline (Fig. 2).

#### Flow Cytometry – Based Prazosin Efflux Assay

The ability of the 11 compounds to inhibit ABCG2-mediated transport at  $10 \mu\text{mol/L}$  was also confirmed by flow cytometry using a different ABCG2 substrate, BODIPY-prazosin. Ten of the 11 compounds tested were active in inhibiting BODIPY-prazosin efflux (1.4- to 5.9-fold BODIPY-prazosin accumulation as compared with 3.5-fold for FTC). Based on relative activities and the availability of a sufficient quantity of materials for additional assays, five of the compounds, NSC11668, NSC19139, NSC120688, NSC168201, and NSC375985 were selected for further testing. Figure 3 (column 1) shows the results of a dose-response assay with BODIPY-prazosin with 0.1, 1, or  $10 \mu\text{mol/L}$  of each of the five compounds. NSC11668 and NSC168201 were the most potent inhibitors as  $0.1 \mu\text{mol/L}$  of these compounds caused the highest increase in intracellular prazosin fluorescence.

#### 5D3 Antibody Binding Assay

To confirm their roles as ABCG2 inhibitors (rather than substrates), these five compounds were next examined for their ability to increase surface staining of the 5D3 antibody (28). Ozvegy-Laczka et al. have previously shown that at high dilution, the 5D3 antibody binds more readily to ABCG2 when ABCG2-transfected cells are incubated with the antibody in the presence of an ABCG2 inhibitor (28). This was believed to be due to the fact that, at low antibody concentrations, 5D3 has a higher affinity for a certain conformation induced by inhibitors of ABCG2, allowing study by flow cytometry. ABCG2-transfected cells were incubated with a high dilution of the 5D3 antibody (1:3,500) in the presence or absence of  $20 \mu\text{mol/L}$  of the putative inhibitor. Cells were subsequently incubated with APC-labeled secondary antibody. Figure 3 (column 2) shows that,

at  $20 \mu\text{mol/L}$ , all of the compounds tested increased 5D3 binding and were comparable to  $20 \mu\text{mol/L}$  of FTC shown as a positive control. The change in APC fluorescence was quantitated for each sample and the values are given in Table 1.

#### Inhibition of Pgp and MRP1

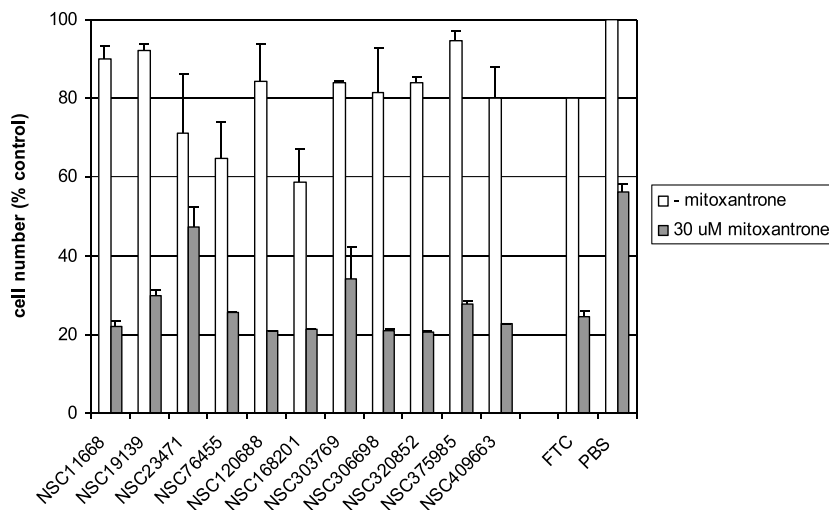
Each of the five selected compounds was also tested for its ability to inhibit Pgp and MRP1, other ABC transporters known to confer drug resistance. None of these compounds significantly inhibited Pgp-mediated rhodamine transport (Fig. 3, column 3). Complete inhibition of Pgp was observed with valspodar (Fig. 3, column 3, bottom histogram). Three compounds (NSC11668, NSC19139, and NSC375985) at  $10 \mu\text{mol/L}$  caused detectable inhibition of MRP1-mediated calcein transport (Fig. 3, column 4). NSC375985 was the most active of these. At higher concentration ( $25 \mu\text{mol/L}$ ), all three compounds inhibited MRP1 (NSC11668, 1.3-fold increase in calcein accumulation; NSC19139, 1.4-fold; and NSC375985, 2.1-fold compared with 1.4-fold for the positive control in this experiment). Neither of the other compounds, NSC120688 and NSC168201, had any detectable effect on MRP1.

#### Inhibition of IAAP Incorporation into ABCG2

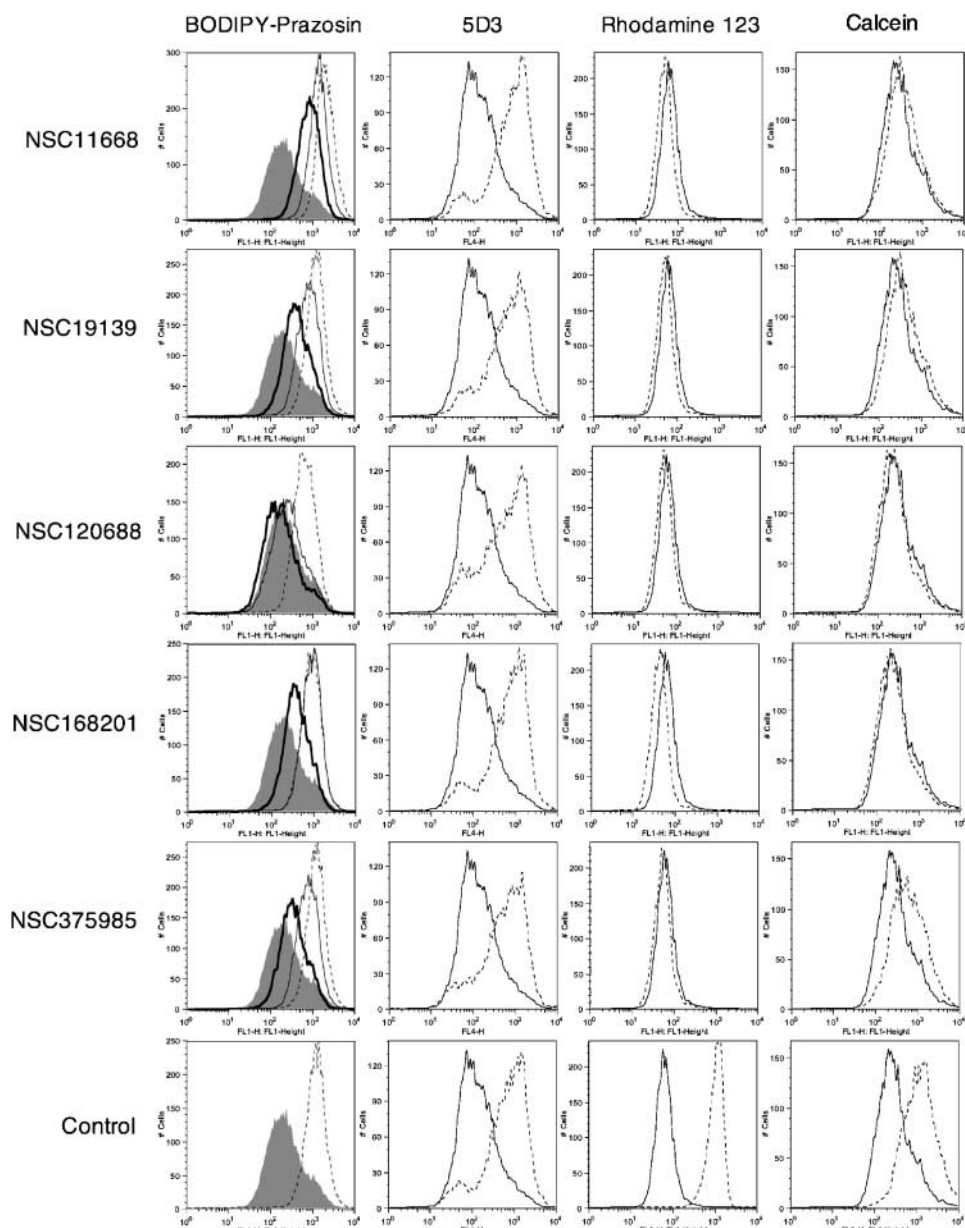
To further explore the interaction between the five compounds and ABCG2, their ability to inhibit [ $^{125}\text{I}$ ]IAAP incorporation into ABCG2 in membranes isolated from ABCG2-overexpressing MCF-7 FLV1000 cells was studied. Previously, IAAP had been shown to be transported by ABCG2 and could also be used as a photoaffinity label for this transporter (29). As seen in Fig. 4, all five compounds (at  $20 \mu\text{mol/L}$ ) significantly reduced [ $^{125}\text{I}$ ]IAAP incorporation into ABCG2. With the exception of NSC375985, all had inhibitory activity comparable to or better than that of FTC. These results suggest that the five compounds act at the binding site of IAAP, similar to FTC.

## Discussion

The NCI-DTP's libraries of synthetic compounds and purified natural products has been an excellent source



**Figure 2.** Sensitization of ABCG2-overexpressing cells to mitoxantrone. The compounds listed were tested for their ability to sensitize NCI-H460/MX20 cells to killing by mitoxantrone as described in Materials and Methods. Cells were treated for 2 d in the presence or absence of  $10 \mu\text{mol/L}$  of compound and  $30 \mu\text{mol/L}$  of mitoxantrone and cell number assessed by the XTT assay (23). Final DMSO concentration was 0.2% (v/v). Cell survival was normalized to buffer control (no mitoxantrone, no compound = 100%). Columns, mean range of duplicate determinations; bars, range.



**Figure 3.** Functional assays of the effects of selected compounds on ABCG2, Pgp, and MRP1. *Column 1*, ABCG2-transfected cells were incubated in BODIPY-prazosin in the absence (*shaded histogram*) or presence of 0.1 (*heavy solid line*), 1 (*solid line*), or 10  $\mu\text{mol/L}$  (*dashed line*) of NSC11668, NSC19139, NSC120688, NSC168201, or NSC375985 as detailed in Materials and Methods. FTC (10  $\mu\text{mol/L}$ , *bottom histogram*) is shown as a positive control for ABCG2 inhibition. *Column 2*, ABCG2-transfected cells were incubated with unlabeled 5D3 antibody (1:3,500) in the absence (*solid line*) or presence (*dashed line*) of 20  $\mu\text{mol/L}$  of the desired compound after which cells were incubated in APC-labeled secondary antibody. FTC (*bottom histogram*) at a concentration of 20  $\mu\text{mol/L}$  is shown as a positive control for Pgp inhibition (*bottom histogram*). *Column 3*, MDR1-transfected cells were incubated in 0.5  $\mu\text{g/mL}$  of rhodamine 123 in the absence (*solid line*) or presence (*dashed line*) of 10  $\mu\text{mol/L}$  of the desired inhibitor as detailed in Materials and Methods. Valspodar at 3  $\mu\text{g/mL}$  is included as a positive control for Pgp inhibition (*bottom histogram*). *Column 4*, MRP1-transfected cells were incubated in 200 nmol/L of calcein AM in the absence (*solid line*) or presence (*dashed line*) of 10  $\mu\text{M}$  of the compounds. MK-571 (25  $\mu\text{mol/L}$ ) is shown (*bottom histogram*) as a positive control for inhibition of MRP1 transport.

of reagents for the study of molecular targets in cancer (19, 20). The application of a high-throughput assay for inhibitors of ABCG2 activity (18) has identified a variety of active compounds. As expected, and previously reported, several flavonoids were among the inhibitors identified which served as a validation for the assay (18). However, as

a class, flavonoids are well characterized as ABCG2 inhibitors (30, 31) and the screening did not identify any novel flavonoids. Therefore, these were not additionally characterized. Based on further analysis, one of the other compounds identified, NSC23471, seemed to be a false-positive result in that it was inactive in alternative assays of

**Table 1.** Summary of effects of compounds in multiple assays

	PhA		MX sensitization*	Flow—ABCG2		IAAP binding <sup>†</sup>	Cross-reactivity (flow)	
	Total <sup>‡</sup>	IC <sub>50</sub> (μmol/L)		5D3 <sup>§</sup>	BODIPY-prazosin <sup>  </sup>		Pgp <sup>¶</sup>	MRP1 <sup>**</sup>
NSC11668	84.1	4.5	21.9	4.0	5.9	24.6	0.73	1.2
NSC19139	76.6	2.6	30.0	3.6	3.5	33.5	0.77	1.3
NSC120688	67.2	4.3	20.9	3.7	2.0	19.1	0.84	0.81
NSC168201	104.5	3.9	21.2	3.7	2.6	34.2	0.64	0.81
NSC375985	63.8	3.7	27.6	3.2	3.6	48.7	0.84	1.8
FTC	100	0.8	24.5	3.7	3.5	32.7		
MK571 (25 μmol/L)								3.3
Valspodar (3 μg/mL)							14.9	
DMSO/PBS	0		56.1	1.0	1.0	100		

\* Percentage NCI-H460/MX20 cell survival in the presence of compound and mitoxantrone.

<sup>†</sup> Blocking of [<sup>125</sup>I]IAAP binding: percentage of control binding in the presence of 20 μmol/L compound.

<sup>‡</sup> Percentage of FTC response.

<sup>§</sup> 5D3 staining: treated/control ratio at 10 μmol/L compound.

<sup>||</sup> BODIPY-prazosin efflux: treated/control ratio at 10 μmol/L compound.

<sup>¶</sup> Pgp inhibition, rhodamine efflux: treated/control ratio at 10 μmol/L compound.

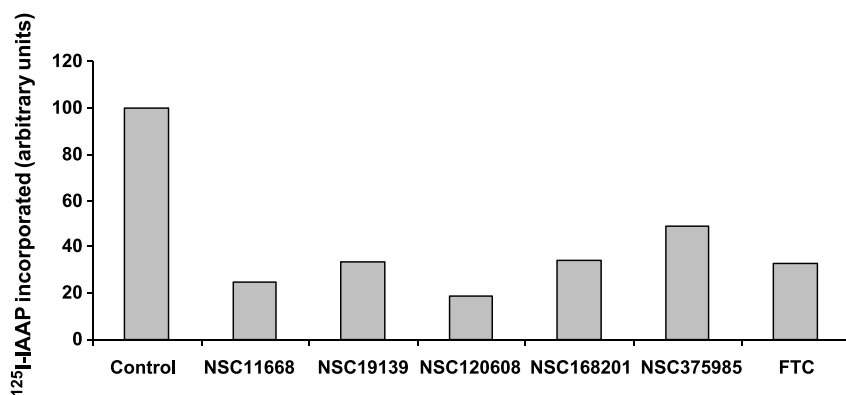
<sup>\*\*</sup> MRP1 inhibition, calcein efflux: treated/control ratio at 10 μmol/L compound.

ABCG2 activity, mitoxantrone sensitization (Fig. 2) and in the BODIPY-prazosin efflux assay (data not shown). Ten new putative inhibitors of ABCG2 have thus been identified. These compounds did not share significant structural features (data not shown) nor did they seem to be similar to previously identified ABCG2 modulators described in the literature.

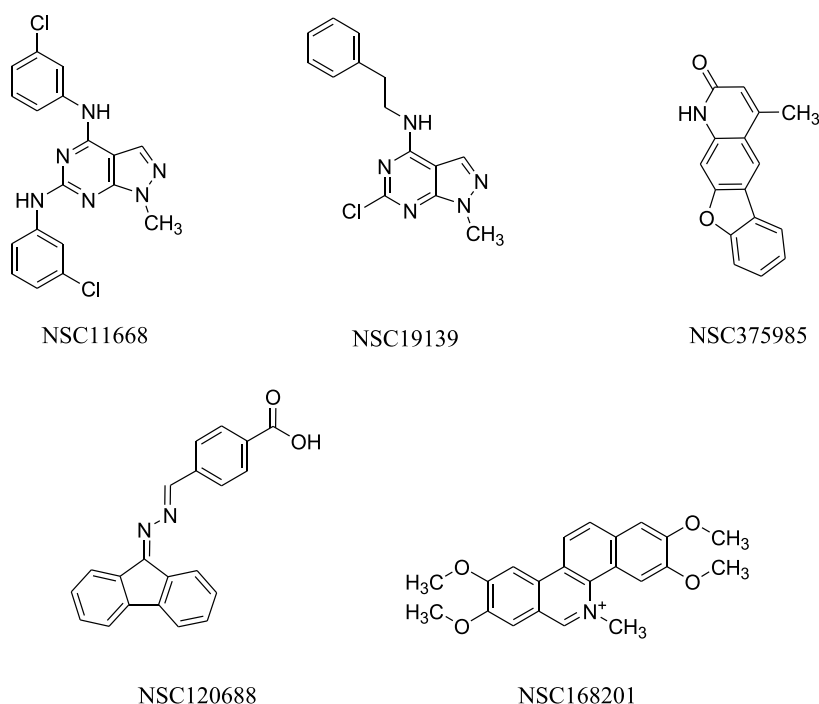
Based on a combination of relative activities in these assays and the availability of sufficient material for further study, five compounds (NSC11668, NSC19139, NSC120688, NSC168201, and NSC375985) were selected for additional characterization. Figure 5 shows the structures of these compounds as reported by the DTP. After resupply of individual compounds from the DTP, their identity and purity were confirmed by liquid chromatography/mass spectrometry and nuclear magnetic resonance analyses (data not shown). Although NSC11668 and NSC19139 share some structural features, the members of this set of compounds are not otherwise related. Table 1 summarizes the data obtained with these five compounds. All five had IC<sub>50</sub> values of <5 μmol/L in the PhA accumulation assay

(Fig. 1) and they were approximately equally potent in the sensitization of cells to mitoxantrone (Fig. 2). Flow cytometry data with an alternative substrate (BODIPY-prazosin) and cell line (ABCG2-transfected HEK293 cells) confirmed the activity of the compounds and gave a preliminary ranking of their relative activities (Fig. 3, column 1). Inhibition of photoaffinity labeling of ABCG2 with [<sup>125</sup>I]IAAP (Fig. 4) confirms the interaction of the compounds with the transporter itself and suggests that these compounds act at the drug substrate-binding site(s). Because IAAP is a photoaffinity analogue of prazosin, it is thought to label the drug-binding site. Although reduced IAAP binding could be due to conformational changes induced by the inhibitor, the more likely explanation is that the decreased binding is due to the competition of the inhibitor for the drug-binding site. Finally, the ability of all five compounds to increase surface staining of the 5D3 antibody confirmed their identification as inhibitors (28).

Comparison of the activities of the five compounds on ABCG2 as compared with two other MDR proteins, Pgp and MRP1, was undertaken. None of the five compounds



**Figure 4.** Effect of selected compounds on photoaffinity labeling of ABCG2 with IAAP. [<sup>125</sup>I]IAAP incorporated into the ABCG2 band was quantified as described in Materials and Methods. Columns, the amount of [<sup>125</sup>I]IAAP incorporated (average of three independent experiments) into ABCG2 (Y-axis) in the absence (control) or presence of 20 μmol/L of the indicated compound (X-axis).



**Figure 5.** Structures of selected ABCG2 inhibitors.

affected Pgp activity. Similarly, NSC120688 and NSC168201 had no effect on MRP1 activity. Among the other compounds, the order of potency against MRP1 was NSC375985 > NSC19139 > NSC11668. However, all of these compounds were much more potent against ABCG2 (see Table 1).

As with other MDR transporters, one of the significant obstacles to development of clinically useful ABCG2 inhibitors is the problem of cytotoxicity (4, 5). The five compounds shown in Fig. 5 all had low toxicity against parental NCI-H460 cells (data not shown). Similarly, a review of NCI 60 cell data in the DTP web site database revealed average GI<sub>50</sub> (50% growth inhibition) of 10 to 70  $\mu\text{mol/L}$  for all 60 cell lines with very little selectivity. These compounds themselves may therefore have relatively low toxicity, but more toxicity studies must be done.

Although the relevance of ABCG2 to clinical drug resistance remains unconfirmed, ABCG2 has been implicated in drug resistance in leukemia (32). The availability of new inhibitors may be able to contribute to increased clinical response in ABCG2-overexpressing tumors. Because ABCG2 has also been reported to be expressed at high levels in the digestive tract and at the blood-brain barrier (33), ABCG2 inhibitors may be able to enhance bioavailability and brain penetration of ABCG2 substrate drugs. Proof of this principle has been shown in a clinical study by Kruitzer et al., who showed that coadministration of GF210918 with topotecan increased the oral absorption of topotecan (34). The compounds identified are somewhat less potent than FTC in the PhA accumulation assay (Table 1). However, all five are considerably more active

than a variety of other known ABCG2 inhibitors in the same assay [novobiocin, estradiol, estrone, quercetin, reserpine, XR9576 (tariquidar), and several other flavonoids; ref. 17]. Identification of any relative advantages or disadvantages of these compounds compared with more potent inhibitors such as the Ko series of FTC derivatives (35) must await *in vivo* efficacy and toxicity studies.

In conclusion, five novel compounds have been identified from the NCI-DTP repository. These compounds are relatively specific inhibitors of the ABCG2 MRP with varying cross-reactivity against MRP1. The compounds identified by this work will provide a selection of novel reagents for studying ABCG2 function and potential clinical relevance as well as potential scaffolds for building newer inhibitors.

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