Inhibition of ABCG2-mediated transport by protein kinase inhibitors with a bisindolylmaleimide or indolocarbazole structure

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Abstract

ABCG2 is a transporter with potential importance in cancer drug resistance, drug oral absorption, and stem cell biology. In an effort to identify novel inhibitors of ABCG2, we examined the ability of commercially available bisindolylmaleimides (BIM) and indolocarbazole protein kinase inhibitors (PKI) to inhibit ABCG2, given the previous demonstration that the indolocarbazole PKI UCN-01 interacted with the transporter. At a concentration of 10 μmol/L, all of the compounds tested increased intracellular fluorescence of the ABCG2-specific substrate pheophorbide a in ABCG2-transfected HEK-293 cells by 1.3- to 6-fold as measured by flow cytometry; the ABCG2-specific inhibitor fumitremorgin C increased intracellular fluorescence by 6.6-fold. In 4-day cytotoxicity assays, wild-type ABCG2-transfected cells were not more than 2-fold resistant to any of the compounds, suggesting that the PKIs are not significantly transported by ABCG2. BIMs I, II, III, IV, and V, K252c, and arcyriaflavin A were also able to inhibit [^{125}I]iodoarylazidoprazosin labeling of ABCG2 by 65% to 80% at 20 μmol/L, compared with a 50% to 70% reduction by 20 μmol/L fumitremorgin C. K252c and arcyriaflavin A were the most potent compounds, with IC_{50} values for inhibition of [^{125}I]iodoarylazidoprazosin labeling of 0.37 and 0.23 μmol/L, respectively. K252c and arcyriaflavin A did not have any effect on the ATPase activity of ABCG2. Four minimally toxic compounds—BIM IV, BIM V, arcyriaflavin A, and K252c—reduced the relative resistance of ABCG2-transfected cells to SN-38 in cytotoxicity assays. We find that indolocarbazole and BIM PKIs directly interact with the ABCG2 protein and may thus increase oral bioavailability of ABCG2 substrates. [Mol Cancer Ther 2007;6(6):1877–85]

Introduction

Protein kinases are currently an attractive target for drug development. Whereas several classes of protein kinase inhibitors (PKI) have been reported, the discovery that staurosporine inhibited protein kinase C (PKC) at nanomolar concentrations spawned the development of indolocarbazole and bisindolylmaleimide (BIM) PKIs with a structure similar to that of staurosporine (1). Indolocarbazoles currently in clincial trials for cancer treatment include UCN-01 and PKC412 (midostaurin, *N*-benzoyl staurosporine), whereas the BIM ruboxistaurin (LY333531) is currently being evaluated as a treatment for diabetic complications (1). PKC412 has shown some antitumor effects alone, but its use is also being explored in combination with cisplatinum and 5-fluorouracil as well as separately as a radiosensitizer (2, 3). The indolocarbazole CEP-701, an FLT3 inhibitor, has shown promise in the treatment of leukemia (4), and enzastaurin is a PKC-β inhibitor currently in clinical trials (5, 6). Preclinical studies have also been conducted with the BIMs Ro 31-8220 (BIM IX) and Ro 32-0432 (BIM XI) and the indolocarbazole ICP-1(7, 8). Structures of some of the compounds in clinical trials are given in Fig. 1.

During their clinical development, indolocarbazoles and BIMs were shown to interact with ATP-binding cassette (ABC) transporters. The indolocarbazoles staurosporine, UCN-01, and PKC412 are known to inhibit P-glycoprotein–mediated drug transport possibly due to competitive inhibition, whereas PKC412 inhibits P-glycoprotein but is not itself transported (9–11). BIMs have also been shown to inhibit P-glycoprotein as well as multidrug resistance–associated protein 1 (MRP1; refs. 12–14). Indolocarbazole topoisomerase I inhibitors such as J-107088 (edotecarin) have been found to be substrates of the ABC half-transporter ABCG2 (15, 16). Our laboratory has previously reported that the indolocarbazole UCN-01 is a substrate and inhibitor of ABCG2 (17). We therefore hypothesized a potential interaction between indolocarbazole and BIM PKIs and ABCG2.

ABCG2 is an ABC half-transporter that has been shown to confer resistance to a wide variety of chemotherapeutic agents including mitoxantrone, SN-38, flavopiridol, and topotecan (18–22). Although the clinical significance of ABCG2 expression in cancer is currently under investigation, immunochemical analysis of tumor samples has

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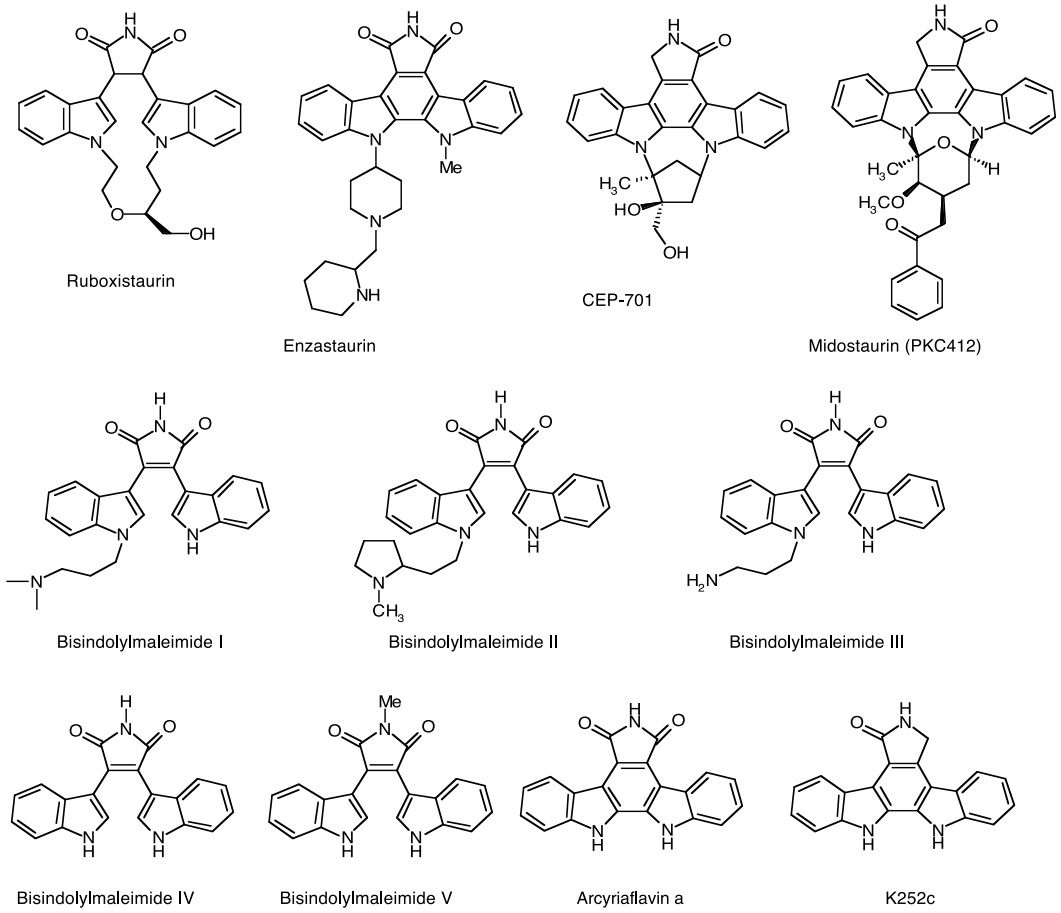

Figure 1. Chemical structures of selected BIMs and indolocarbazoles.

shown expression of ABCG2 in adenocarcinomas of the digestive tract, endometrium, and lung as well as melanoma (23). Recent studies have also linked ABCG2 expression to shorter duration of remission (24) and poor prognosis (25) in acute myeloid leukemia. Expression of ABCG2 in normal tissues is highest in the placenta, but is also found at high levels in the brain, where it is believed to be a component of the blood-brain barrier; in the gut, where it is believed to mediate oral absorption of drugs; and in hematopoietic stem cells, where it is the determinant of the side population and may protect stem cells from xenobiotics (18, 26–30). ABCG2 expression has also been noted in the prostate, ovary, and liver (31, 32). Thus, it will be important to characterize the interaction of oral anticancer agents as well as nononcologic agents with ABCG2.

Herein we report the interaction of ABCG2 with indolocarbazole or BIM PKIs. Among the PKIs examined, all were found to inhibit ABCG2, but none were themselves appreciably transported. The results presented here suggest that indolocarbazole or BIM PKIs could overcome drug resistance in ABCG2-overexpressing tumors or provide a means to deliver molecularly targeted therapy to cancer stem cells if such are ultimately found to play a role in drug resistance. Indolocarbazoles and BIMs represent novel classes of ABCG2 inhibitors.

Materials and Methods

Chemicals

The PKIs BIM I, II, III, IV, V, VIII, IX, X, and XI, Go6983, arcyriaflavin A, KT5720, KT5823, Go6976, Go7874, K252a, K252c, and *N*-benzoyl staurosporine (PKC412), as well as the cyclin-dependent kinase inhibitors 2-bromo-12,13-dihydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*)-dione (CDK4I) and SB-218078, were purchased from EMD Biosciences. BIM VI and BIM VII were purchased from Alexis Biochemicals. The chemical structures of selected compounds are given in Fig. 1. Pheophorbide a (PhA) was obtained from Frontier Scientific. The ABCG2-specific inhibitor fumitremorgin C was isolated by Thomas McCloud (Developmental Therapeutics Program, Natural Products Extraction Laboratory, NIH, Bethesda, MD). [^{125}I]Iodoarylazidoprazosin (IAAP; 2,200 Ci/mmol/L) was purchased from Perkin-Elmer Life Sciences.

Cell Lines

Human embryonic kidney (HEK-293) cells stably transfected with empty pcDNA3.1 vector or pcDNA3.1 vector containing full-length, wild-type *ABCG2* (R-5) have previously been characterized (33). ABCG2-overexpressing MCF-7 FLV1000 cells were grown in Richter's medium with 10% FCS and were additionally maintained in 1,000 nmol/L flavopiridol (19).

Flow Cytometry

Flow cytometry assays were done as previously described, with some modification (17). Briefly, cells were trypsinized and incubated for 30 min at 37°C and 5% CO_2 in complete medium (phenol red–free Richter's medium with 10% FCS) containing 1 μmol/L PhA, a concentration which affords greater sensitivity, with or without 10 μmol/L of the ABCG2 inhibitor fumitremorgin C or the potential inhibitor being tested. Cells were then incubated for 1 h at 37°C in PhA-free medium continuing with or without fumitremorgin C or potential inhibitor to generate the Inhibitor/Efflux and Efflux histograms, respectively. To obtain the fold increase in PhA fluorescence, the mean value of the Inhibitor/Efflux histogram (in log units) was divided by the mean value of the Efflux histogram (in log units).

To determine the effect of the prospective inhibitors on 5D3 antibody binding, we used the methods of Ozvegy-Laczka et al. (34), with minor modifications. ABCG2-overexpressing R-5 cells were incubated in a 1:3,500 dilution of unlabeled 5D3 antibody (eBioscience) in the presence or absence of 20 μmol/L of the desired inhibitor or 10 μmol/L fumitremorgin C for 2 h in 2% bovine serum albumin. The dilution of 5D3 antibody was obtained by titering the antibody for maximum shift in the presence of the known ABCG2 inhibitor fumitremorgin C. Subsequently, the cells were washed and then incubated with allophycocyanin-labeled anti-mouse secondary antibody (1:35; Leinco Technologies, Inc.).

PhA and allophycocyanin fluorescence was detected on a FACSort flow cytometer (Becton Dickinson) equipped with a 635-nm red diode laser and a 561-nm band-pass filter. For all samples, at least 10,000 events were collected. Debris was eliminated by gating on forward scatter versus side scatter and dead cells were excluded based on propidium iodide staining.

Cytotoxicity Assays

Four-day cytotoxicity assays were done using the sulforhodamine B assay (33). Cells were plated in flat-bottomed 96-well plates. at a density of 2,500 per well and allowed to attach for 24 h at 37°C in 5% CO_2. Chemotherapeutic agents at various concentrations were added to the cells and the plates were allowed to incubate for 96 h at 37°C in 5% CO_2. Cells were subsequently fixed in 50% trichloroacetic acid. Plates were then washed, dried, and stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid). Absorbances were read on a Bio-Rad plate reader at 540 nm. Each concentration was tested in quadruplicate and controls were done in replicates of eight. Relative resistance values were obtained by dividing the IC_{50} value of the *ABCG2*-transfected clone for each drug by the IC_{50} value for the empty-vector pcDNA3-10 clone.

Photo-Cross-Linking of ABCG2 with [^{125}I]IAAP

Competition of ABCG2 photolabeling with [^{125}I]IAAP was done as previously described (35). Briefly, crude membranes from MCF-7 FLV1000 cells were prepared as described elsewhere (36). The crude membranes (1 mg protein/mL) were incubated with 20 μmol/L of the PKIs or 20 μmol/L fumitremorgin C for 10 min at room temperature in 50 mmol/L Tris-HCl (pH 7.5), to which 3 to 6 nmol/L [^{125}I]IAAP (2,200 Ci/mmol) was added and incubated for an additional 5 min under subdued light. Samples were then exposed to a UV lamp (365 nm) for 10 min at room temperature (21–23°C) to cross-link the radioactive IAAP to ABCG2. Labeled ABCG2 was then immunoprecipitated with 10 μg of BXP-21 antibody (Kamiya Biomedical) as previously described (35). The incorporation of [^{125}I]IAAP into the ABCG2 band was quantified using a STORM 860 phosphor imager system (Molecular Dynamics) with ImageQuaNT software.

ATPase Assay

The ATPase assay was done as previously described with minor modifications (37). Crude membranes from R-5 and pcDNA3-10 cells (100 μg protein/mL) were incubated with varying concentrations of BIM or indolocarbazole inhibitors in the presence or absence of 0.2 mmol/L beryllium sulfate and 2.5 mmol/L sodium fluoride (BeFx) in ATPase assay buffer [50 mmol/L KCl, 5 mmol/L sodium azide, 2 mmol/L EGTA, 10 mmol/L MgCl2, 1 mmol/L DTT, 50 mmol/L MOPS (pH 7.5)] for 5 min at 37°C. The reaction was started by the addition of 5 mmol/L ATP and was stopped by the addition of 0.1 mL of 5% SDS solution. The amount of inorganic phosphate released and the BeFx-sensitive ATPase activity of ABCG2 were determined as previously described (37).

Results

Indolocarbazoles and BIMs Are Inhibitors but not Substrates of ABCG2

To screen for the ability of indolocarbazoles or BIMs to inhibit ABCG2, wild-type ABCG2-transfected HEK-293 cells were incubated in 1 μmol/L PhA alone (*solid line*) or in the presence of 10 μmol/L fumitremorgin C or the indolocarbazole or BIM compounds (*dashed line*). At 10 μmol/L, all of the compounds were able to inhibit ABCG2-mediated efflux of PhA and increase intracellular fluorescence to varying degrees (Table 1). Representative histograms for a selected subset of the compounds are shown in Fig. 2. BIM III and BIM VII were the least effective at preventing ABCG2-mediated PhA transport. BIM V and Go6976 were the most potent BIM inhibitors, whereas SB-218078, arcyriaflavin A, K252a, and K252c were the most potent indolocarbazole inhibitors. Complete inhibition of ABCG2-mediated PhA transport by 10 μmol/L fumitremorgin C is shown as a comparison. Because the structurally related cyclin-dependent kinase inhibitors CDK4I and SB-218078 were able to abrogate ABCG2-mediated PhA transport, inhibition of ABCG2 by BIM and indolocarbazole compounds does not seem to be related to inhibition of PKC.

Four-day cytotoxicity assays were subsequently done to determine whether these compounds were ABCG2 substrates and thus presumably acting as competitive inhibitors of ABCG2. IC_{50} values for each of the PKIs were determined for empty vector–transfected HEK-293 cells (pcDNA3-10) or wild-type (R-5) transfected HEK-293 cells. The results are summarized in Table 2. *ABCG2*-transfected cells were not more than 2-fold resistant to any of the compounds compared with empty vector–transfected cells, and in some cases were more sensitive, suggesting that BIMs and indolocarbazoles are not ABCG2 substrates. HEK-293 cells expressing wild-type ABCG2 (R-5) were highly resistant to mitoxantrone (56-fold), consistent with earlier reports (33).

PKIs Increase Binding of the Anti-ABCG2 Antibody 5D3

We then chose the compounds BIMs I, II, III, IV, and V as a representative set of BIM compounds as well as two of the most potent indolocarbazole compounds, K252c and arcyriaflavin A, and examined them for their ability to increase binding of the anti-ABCG2 antibody 5D3. Ozvegy-Laczka has 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 (34). 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 (34). *ABCG2*-transfected R-5 cells were incubated with 5D3 antibody (1:3,500) in the presence (*dashed line*) or absence (*solid line*) of 20 μmol/L of the desired compound, after which the cells were incubated with allophycocyanin-labeled secondary antibody (1:35). As seen in Fig. 2B, known inhibitors of ABCG2, fumitremorgin C (20 μmol/L) and high concentrations of flavopiridol (50 μmol/L), increased labeling of the 5D3 antibody as previously shown by Ozvegy-Laczka (34). The known substrate of ABCG2, topotecan (10 μmol/L), did not appreciably increase binding of the 5D3 antibody. The substrates mitoxantrone and SN-38 also did not increase 5D3 binding (data not shown). All seven of the PKIs examined increased 5D3 labeling. These results support the hypothesis that BIM and indolocarbazole PKIs behave as ABCG2 inhibitors, possibly with a mechanism of action similar to that of fumitremorgin C.

[^{125}I]IAAP Labeling of ABCG2 Is Inhibited by Indolocarbazole and BIM Compounds

To further explore the interaction between indolocarbazole and BIM PKIs, BIMs I, II, III, IV, and V, K252c, and arcyriaflavin A were again selected and examined for their ability to prevent [^{125}I]IAAP labeling of ABCG2 in membranes isolated from MCF-7 FLV1000 cells. As seen in Fig. 3A, all of the compounds tested reduced [^{125}I]IAAP labeling of ABCG2 to levels 20% to 40% of the control at a concentration of 20 μmol/L, thus confirming an interaction between the PKIs and ABCG2. The ABCG2-specific inhibitor fumitremorgin C (20 μmol/L) was included as a positive control, inhibiting [^{125}I]IAAP labeling by ~60%. These results suggested that BIM and indolocarbazole PKIs act at the drug binding site of ABCG2. For the most potent of the inhibitors tested, K252c and arcyriaflavin A, we carried out a dose-response study, examining [^{125}I]IAAP labeling of ABCG2 at various inhibitor concentrations (data not shown). The IC_{50} values for K252c and arcyriaflavin A were found to be 0.37 and 0.23 μmol/L, respectively. This can be contrasted to results we previously obtained, in which fumitremorgin C prevented [^{125}I]IAAP labeling of ABCG2 with an IC_{50} value of ~5 μmol/L in membranes from MCF-7 FLV1000 cells (35).

Table 1. Fold increase in intracellular PhA fluorescence

Compound	Fold increase (±SD)*
Fumitremorgin C	6.6 ± 3.3
BIM I	2.8 ± 1.1
BIM II	2.7 ± 1.1
BIM III	1.6 ± 0.6
BIM IV	2.8 ± 1.0
BIM V	4.1 ± 1.7
BIM VI	2.3 ± 1.1
BIM VII	1.3 ± 0.1
BIM VIII	2.1 ± 0.8
BIM IX	1.7 ± 0.6
BIM X	1.9 ± 0.5
BIM XI	1.7 ± 0.6
Go6976	5.3 ± 2.0
Go7874	3.2 ± 1.1
K252a	6.0 ± 2.5
K252c	5.1 ± 2.1
KT5823	4.0 ± 1.0
Arcyriaflavin A	5.8 ± 3.3
PKC412	2.5 ± 0.7
CDK4I	2.9 ± 1.2
SB-218078	5.7 ± 3.3

NOTE: All compounds were tested at a concentration of 10 μmol/L.
*Fold increase in intracellular fluorescence was determined by dividing the mean fluorescence value of the Inhibitor/Efflux histogram (in log units) by the mean fluorescence value of the Efflux histogram (in log units). Results are from at least two independent experiments.

ATPase Activity of ABCG2 Is Unaffected by K252c and Arcyriaflavin A

Some substrates of ABC transporters are known to stimulate the ATPase activity of the transporter, and it has been shown that ABCG2 substrates such as mitoxantrone and prazosin increase ATPase activity of the protein whereas ABCG2 inhibitors such as Ko143 decrease ATPase activity (38). However, such strict correlations do not always hold true for P-glycoprotein substrates. The two most potent inhibitors of ABCG2 in our study, arcyriaflavin A and K252c, were assessed for their ability to affect the ATPase activity of ABCG2. As shown in Fig. 3B, nifedipine, a known substrate of ABCG2, stimulated the ATPase activity of ABCG2 by ~4-fold. In contrast, K252c and arcyriaflavin A did not appreciably affect ATPase activity of ABCG2.

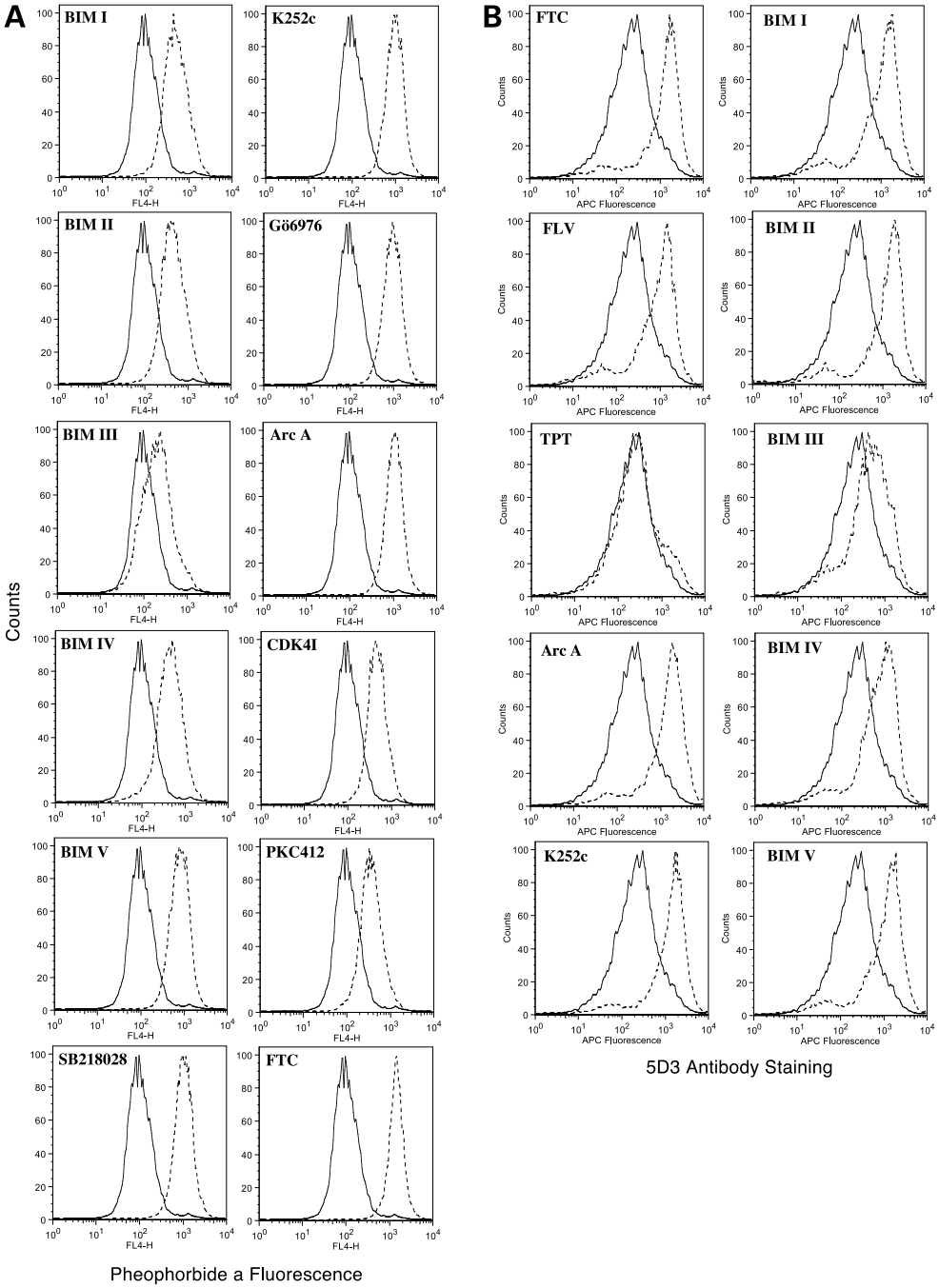

Figure 2. BIM and indolocarbazole kinase inhibitors abrogate ABCG2-mediated PhA transport and increase 5D3 binding. **A,** ABCG2-transfected R-5 cells were incubated with 1 μmol/L PhA in the presence or absence of 10 μmol/L of the indicated potential inhibitor for 30 min at 37°C, washed, then allowed to incubate in PhA-free medium for 1 h continuing with (*dashed line*) or without (*solid line*) inhibitor. Representative histograms for the compounds BIMs I, II, III, IV, and V, SB-218048, K252c, Go6976, arcyriaflavin A (*Arc A*), CDK4I, and PKC412 from at least two independent experiments are shown. The known ABCG2 inhibitor fumitremorgin C (*FTC*) is included as a positive control. **B,** ABCG2-transfected cells were incubated with a 1:3,500 dilution of unlabeled 5D3 antibody in the presence (*dashed line*) or absence (*solid line*) of the desired compound, washed, and subsequently incubated in a 1:100 dilution of allophycocyanin-labeled secondary antibody. Representative of at least two separate experiments. *FLV,* flavopiridol; *TPT,* topotecan.

Indolocarbazoles and BIMs Abrogate ABCG2-Mediated SN-38 Resistance

BIM IV, BIM V, K252c, and arcyriaflavin A were then selected for further study because we were able to administer nontoxic doses of the drugs and still inhibit ABCG2-mediated transport. Based on the cytotoxicity data (summarized in Table 2), ~80% of empty vector–transfected (pcDNA3-10) and ABCG2-transfected cells

Table 2. Cross-resistance of ABCG2-transfected cells to BIM or indolocarbazole compounds

Compound	IC_{50} pcDNA3-10 (±SD), μmol/L	IC_{50} R-5 (±SD), μmol/L	Relative resistance*
MX	0.0025 ± 0.0007	0.14 ± 0.08	56
BIM I	5.5 ± 0.7	5.5 ± 0.7	1
BIM II	5.5 ± 0.7	5.5 ± 0.7	1
BIM III	5.6 ± 2.5	10.3 ± 0.6	2
BIM IV	10.7 ± 0.4	10.7 ± 0.4	1
BIM V	10.5 ± 0.7	10.5 ± 0.7	1
BIM VI	3.2 ± 3.2	3.2 ± 3.2	1
BIM VII	12.5 ± 4	12.5 ± 4	1
BIM VIII	6.5 ± 0.7	6.5 ± 0.7	1
BIM IX	3 ± 1.7	7.3 ± 2.3	2
BIM X	3.7 ± 0.6	3.5 ± 0.9	1
BIM XI	5.5 ± 0.7	6.5 ± 0.7	1
Go6976	0.04 ± 0.1	0.08 ± 0.007	2
Go7874	0.6 ± 0.3	1.2 ± 0.5	2
K252a	0.4 ± 0.3	0.5 ± 0.07	1
K252c	7.3 ± 4.6	4.7 ± 0.6	1
KT5720	7.0 ± 2.8	7.5 ± 2.1	1
KT5823	10.2 ± 0.4	5.5 ± 0.7	1
Arcyriaflavin A	52.5 ± 3.5	52.5 ± 3.5	1
PKC412	2.0 ± 1.8	3.7 ± 2.5	2
CDK4I	1.1 ± 0.4	1.3 ± 0.6	1
SB-218078	0.4 ± 0.1	0.6 ± 0.1	1

*Relative resistance values were determined by dividing the IC_{50} value for the R-5 cell line by the IC_{50} value for the pcDNA3-10 cell line. Results are from at least two independent experiments.

(R-5) survived a 4-day treatment with 5 μmol/L BIM IV, BIM V, or arcyriaflavin A, or 2 μmol/L K252c. Therefore, we did cytotoxicity assays with SN-38 in the presence of 5 μmol/L of BIM IV, BIM V, or arcyriaflavin A, or 2 μmol/L K252c. Combination studies were carried out in pcDNA3-10 and R-5 cells. At a concentration of 5 μmol/L, BIM V was slightly more effective at inhibiting ABCG2-mediated SN-38 resistance than 5 μmol/L BIM IV, a finding that is also in agreement with flow cytometry data. Both arcyriaflavin A (5 μmol/L) and K252c (2 μmol/L) were found to more potently inhibit ABCG2-mediated SN-38 resistance, also in agreement with flow cytometry data. A summary of the results obtained is presented in Table 3. The ABCG2-transfected R-5 cells were 66-fold resistant to SN-38, a result that is consistent with our previous findings (33).

Combination studies were also attempted with mitoxantrone. However, arcyriaflavin A and K252c both induced increased resistance to mitoxantrone (up to 5-fold) in empty vector–transfected cells (data not shown). BIM IV and BIM V did not have this effect on empty vector–transfected cells and did slightly reverse resistance to mitoxantrone in R-5 cells. As both arcyriaflavin A and K252c potently inhibit the cyclin D/CDK4 complex (39), our data suggest that this complex may play a role in cellular resistance to mitoxantrone, perhaps through a decrease in growth rate due to the schedule dependence of mitoxantrone.

The dose modifying factor was also calculated for each of the inhibitors with SN-38 by dividing the IC_{50} for each drug in the absence of inhibitor by the IC_{50} in the presence of inhibitor. The dose modifying factors for BIM IV, BIM V, K252c, and arcyriaflavin A were 3.3, 7.6, 30, and 58, respectively. Arcyriaflavin A nearly completely abrogated resistance to SN-38 in R-5 cells.

Discussion

Expression of ABC proteins has been linked to drug resistance in cancer, and we remain convinced that the development of effective inhibitors has the potential to increase clinical efficacy of anticancer agents in selected settings. ABCG2, the most recently discovered ABC protein, represents a new target for cancer treatment and drug resistance reversal. Following our earlier studies with the PKI UCN-01, wherein we found it to interact with ABCG2, we examined PKIs with a BIM or indolocarbazole structure for their ability to inhibit ABCG2. All compounds studied inhibited ABCG2-mediated PhA transport at a concentration of 10 μmol/L. Four minimally toxic compounds, BIM IV, V, arcyriaflavin A, and K252c, were able to reverse ABCG2-mediated resistance to SN-38 in transfected cell lines. Arcyriaflavin A and K252c, the most potent inhibitors examined, were able to compete IAAP labeling with an IC_{50} of 0.37 and 0.23 μmol/L, respectively. Thus, these two compounds were the most desirable candidates for reversal of drug resistance because they were minimally toxic themselves but were the most potent inhibitors. They may also be useful to increase oral bioavailability of ABCG2 substrates.

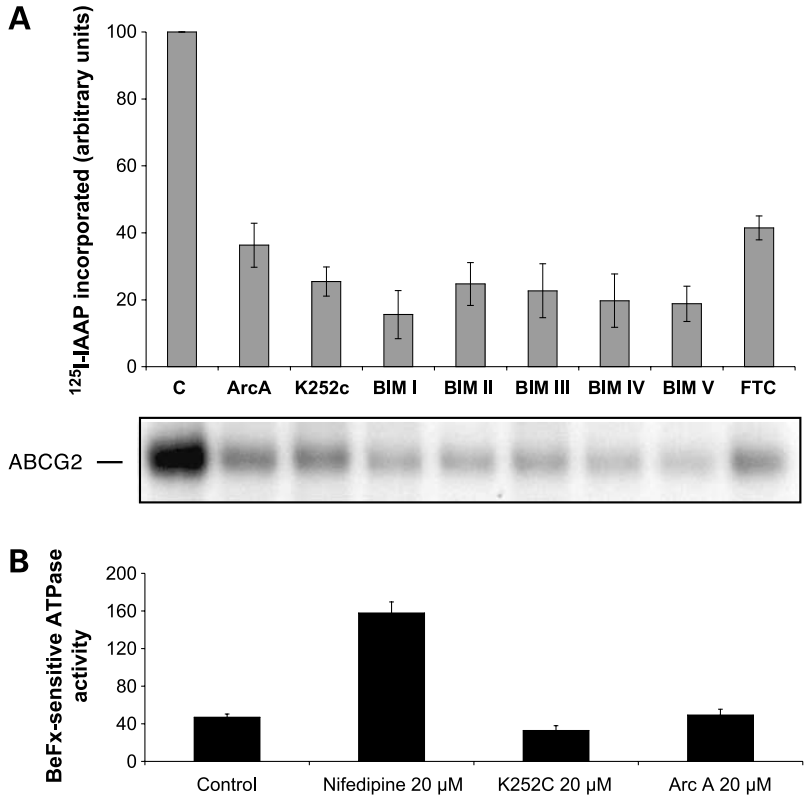

Figure 3. BIM and indolocarbazole kinase inhibitors compete [^{125}I]IAAP binding to ABCG2 but do not increase ATPase activity. **A,** crude membranes isolated from MCF-7 FLV1000 cells were incubated with 20 μmol/L of the desired compound in the presence of [^{125}I]IAAP followed by UV cross-linking to the protein as described in Materials and Methods. Fumitremorgin C is shown as a positive control. An autoradiogram from a representative experiment. The radioactivity incorporated into the ABCG2 band was quantified as described in Materials and Methods. The graph represents a summary of two independent experiments. **B,** the ATPase activity of ABCG2 was determined, as outlined in Materials and Methods, in the presence of 20 μmol/L of the desired compound. Nifedipine was included as a positive control for stimulation of ATPase activity. *Columns,* mean with 90% confidence interval; *bars,* SD ($P < 0.05$; $n = 4$ for each group).

Although we did not carry out an exhaustive structure-activity relationship study, we can draw some conclusions based on our results. Based on inhibition of PhA transport, indolocarbazoles were the most effective. Among the most potent indolocarbazoles were those compounds lacking any sugar moiety attached to the indolocarbazole core, although this was not true for K252a. It is interesting to note that the compounds NB-506, J-107088, and compound A have been shown to be substrates of ABCG2 in cell line models (18), wherein none of the compounds tested here were found to be substrates. In the case of NB-506, J-107088, and compound A, the sugar moiety is attached to only one of the indole nitrogens of the indolocarbazole core. This factor may therefore be important in determining transport by ABCG2.

Because BIM and indolocarbazole PKIs are already in clinical trials for cancer treatment, their transition into use as ABCG2 inhibitors may be relatively easy. As mentioned earlier, the BIM PKC412 is a PKC inhibitor, but it has also been identified as an FLT3 inhibitor (40) along with the compound CEP-701 (4). In a phase II trial of PKC412, plasma concentrations of the parent compound as high as 4 μmol/L have been reported (41). Interestingly, serum concentrations of metabolites of PKC412, also indolocarbazoles, have been reported to be as high as 22 μmol/L (41), thus suggesting that the metabolites might also act as inhibitors of ABCG2. Plasma concentrations of CEP-701 that fall into the range where indolocarbazoles show ABCG2 inhibitory activity have been reported in phase I trials (42). Combination therapies with PKIs currently in clinical trials and ABCG2 substrate drugs may improve drug efficacy. Synergistic effects have been reported when PKC412 was coadministered with mitoxantrone in cell lines harboring FLT3 mutations (43).

Table 3. BIM IV, BIM V, K252c, and arcyriaflavin A reverse ABCG2-mediated resistance to SN-38

Drug	IC_{50} pcDNA3-10 (±SD)	IC_{50} R-5 (±SD)	Dose modifying factor
SN-38	0.0029 ± 0.0017	0.19 ± 0.11	
SN-38 + 5 μmol/L BIM IV	0.0017 ± 0.001	0.058 ± 0.05	3.3
SN-38 + 5 μmol/L BIM V	0.0033 ± 0.0011	0.025 ± 0.0071	7.6
SN-38 + 2 μmol/L K252c	0.0028 ± 0.0010	0.0068 ± 0.0032	30
SN-38 + 5 μmol/L arcyriaflavin A	0.0013 ± 0.00058	0.0033 ± 0.0021	58

NOTE: The dose modifying factor was calculated by dividing the IC_{50} of the R-5 cells for SN-38 in the absence of inhibitor by the IC_{50} in the presence of inhibitor. Results are from at least three independent experiments.

Although the contribution of ABCG2 to clinical drug resistance is currently under investigation, several studies have reported that ABCG2 expression may play a role in drug resistance in leukemia (25, 44). Additionally, Willman et al. (45) recently published results from a microarray analysis suggesting that transporter expression is a marker of poor clinical outcome in acute myeloid leukemia. This is of particular interest for regimens that include mitoxantrone. Inclusion of an ABCG2 inhibitor in the treatment regimen for acute myeloid leukemia may therefore increase response to chemotherapy. Additionally, as the expression of the transporters P-glycoprotein and MRP1 has been reported in leukemia (46), and as BIM compounds are known to also inhibit P-glycoprotein and MRP1 (13, 14), it may be possible to develop compounds that potently inhibit all three transporters. The importance in solid tumors is not yet known because few detailed studies have appeared.

Several researchers have reported high levels of ABCG2 in the digestive tract (47), suggesting a role for ABCG2 in the oral bioavailability of substrate drugs. This has been reported even for drugs currently in clinical development (48). To increase oral bioavailability, coadministration of ABCG2 substrate drugs with ABCG2 inhibitors has been suggested. A clinical study has already provided proof-of-concept for this strategy (30). Thus, PKIs with a BIM or indolocarbazole structure may be used to inhibit ABCG2 and increase the oral bioavailability of substrate drugs.

The 5D3 shift assay, initially reported by Ozvegy-Laczka et al., was used to show that the putative inhibitors were acting as inhibitors of ABCG2. Ozvegy-Laczka et al. (34) found that compounds that inhibited or decreased the ATPase activity of ABCG2 increased 5D3 binding when the antibody was used at low concentrations. Their results suggest that the antibody may recognize a conformation induced by inhibitors or ABCG2 (34). Studies to determine the molecular basis for this phenomenon are ongoing. The assay may be useful in screening for inhibitors of ABCG2 in future studies.

In summary, PKIs with a BIM or indolocarbazole structure represent novel classes of ABCG2 inhibitors. Structure-activity relationship studies may be able to identify inhibitors capable of inhibiting ABCG2, P-glycoprotein, and MRP1 simultaneously.

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