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ORIGINAL ARTICLE

Becatecarin (rebeccamycin analog, NSC 655649) is a transport substrate and induces expression of the ATP-binding cassette transporter, ABCG2, in lung carcinoma cells

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Abstract
Purpose ABCG2 overexpression has been linked to resistance to topoisomerase inhibitors, leading us to examine the potential interaction between ABCG2 and becatecarin.
Methods Interaction with ABCG2 was determined by ATPase assay, competition of [^{125}I]iodoarylazidoprazosin (IAAP) photolabeling and flow cytometry. Cellular resistance was measured in 4-day cytotoxicity assays. ABCG2 expression was measured by fluorescent-substrate transport assays and immunoblot.
Results Becatecarin competed [^{125}I]-IAAP labeling of ABCG2, stimulated ATPase activity and, at concentrations greater than 10 μM, inhibited ABCG2-mediated transport. Becatecarin-selected A549 Bec150 lung carcinoma cells were 3.1-, 15-, 8-, and 6.8-fold resistant to becatecarin, mitoxantrone, SN-38 and topotecan, respectively. A549 Bec150 cells transported the ABCG2 substrates pheophorbide a, mitoxantrone and BODIPY-prazosin and displayed increased staining with the anti-ABCG2 antibody 5D3 compared to parental cells. Increased ABCG2 expression was confirmed by immunoblot.
Conclusions Our results suggest that becatecarin is transported by ABCG2 and can induce ABCG2 expression in cancer cells.

Keywords Becatecarin · Rebecamycin derivative · ABCG2 · Drug-resistance · Topoisomerase inhibitor

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Introduction

Becatecarin (rebeccamycin derivative, NSC 655649) is a diethylaminoethyl analog of rebeccamycin. The parent compound, rebeccamycin, was isolated from the actinomycete *Saccharothrix aerocoligenes* and was shown to inhibit topoisomerase I and II, but its poor water solubility precluded its clinical use, leading to the synthesis of the water-soluble analog, becatecarin. Early clinical studies with becatecarin were promising, with reports from phase I studies noting partial responses in patients diagnosed with gastric cancer [1] or adenocarcinoma of unknown origin [2], and minor responses in patients with gall bladder or pancreatic tumors [1]. In contrast, in phase II studies in patients with breast [3], renal cell [4] or colorectal cancer [5], becatecarin displayed relatively modest activity. When becatecarin was combined with cisplatin, partial responses were noted in patients with adenocarcinoma of unknown origin, non-small cell lung cancer, and pancreatic cancer; however, progressive disease was eventually reported in all patients [6]. In a phase II study of becatecarin in children with solid tumors, only 4 of 126 patients saw clinical benefit and the investigators noted significant myelosupression [7].

Other indolocarbazole topoisomerase inhibitors with structures similar to becatecarin have been developed. NB-506 is a

glycosylated derivative of BE-13793C, an indolocarbazole topoisomerase I and II inhibitor isolated from the fermentation broth of *Streptoverticillum morbaraense* [8]. The NB-506 derivative edotecarin (J-107088) was found to have even greater potency and activity than the parent compound [9]. Preclinical studies with the compounds revealed that NB-506 was not subject to transport by the ATP-binding cassette (ABC) transporters P-glycoprotein (Pgp) or multidrug resistance associated protein 1 (MRP1) [10], and that edotecarin (J-107088) was not found to be transported by Pgp [11]. Komatani and colleagues, however, found that both compounds were subject to transport by the ABC half-transporter ABCG2 [12]. Given the similarity in structure between NB-506, edotecarin and becatecarin, we decided to examine the interaction between becatecarin and ABCG2.

Overexpression of ABCG2 in cell line models has been found to be associated with resistance to several antineoplastics including mitoxantrone, flavopiridol, and the camptothecin derivatives topotecan, SN-38 and 9-aminocamptothecin [13, 14]. Elevated levels of ABCG2 are found in the placenta, brain endothelium and gastrointestinal tract, leading to the theory that ABCG2 forms part of the maternal–fetal barrier, the blood–brain-barrier and also plays a role in oral drug absorption [13]. Although its contribution to clinical drug resistance is currently under investigation, several studies conclude that ABCG2 does play a role in drug resistance in acute myelogenous leukemia [15].

In the present work, we sought to characterize the interactions between becatecarin and ABCG2. We found it to be a weak substrate for ABCG2 and, at high concentrations, becatecarin was found to act as an ABCG2 inhibitor. A becatecarin-resistant A549 lung carcinoma subline was developed and was found to overexpress ABCG2 as its mechanism of resistance. Our results suggest that becatecarin may induce ABCG2 in tumors leading to increased resistance to other ABCG2 substrate drugs.

Materials and methods

Chemicals

Becatecarin (rebeccamycin derivative, NSC 655649) was obtained from the National Cancer Institute Anticancer Drug Screen (Bethesda, MD, USA). Mitoxantrone was obtained from Sigma Chemical (St Louis, MO, USA). Topotecan and SN-38 were purchased from LKT Laboratories (St. Paul, MN, USA). BODIPY-prazosin was acquired from Invitrogen Corporation (Carlsbad, CA, USA). Pheophorbide a was purchased from Frontier Scientific (Logan, UT). The ABCG2-specific inhibitor fumitremorgin C (FTC) was isolated by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, National Institutes of Health (Bethesda, MD, USA). [^{125}I]iodoarylazidoprazosin ([^{125}I]-IAAP, 2,200 Ci/mM) was obtained from Perkin Elmer Life Sciences (Wellesley, MA, USA).

Cell lines

Parental A549 non-small cell lung carcinoma cells (obtained from the NCI anticancer drug screen) were exposed to incrementally increasing amounts of becatecarin to generate the A549 Bec150 subline, which is maintained in 150 nM becatecarin. ABCG2-overexpressing MCF-7 MX and MCF-7 FLV1000 cells are maintained in Richter's medium with 100 nM mitoxantrone [16] or 1,000 nM flavopiridol [17], respectively. Mitoxantrone-selected SF295 MX500 cells are maintained in 500 nM mitoxantrone [18]. Empty vector-(pcDNA3.1) and wild-type *ABCG2*-transfected (R-2) as well as *ABCB1*-transfected (MDR-19) and *ABCC1*-transfected (MRP1) human embryonic kidney cells (HEK293) were maintained in Eagle's medium with G418 at a concentration of 2 mg/ml [19].

Immunoblot

An immunoblot assay for Pgp, MRP1 and ABCG2 was performed on microsomal membrane protein using the C219 (Signet Laboratories, Dedham, MA, USA), MRPm6 (Kamiya Biomedical, Seattle, WA, USA) and BXP-21 (Kamiya Biomedical) antibodies, respectively, as previously described [20].

Flow cytometry

Flow cytometry assays with the ABCG2 substrates BODIPY-prazosin, mitoxantrone and pheophorbide a were performed [16, 20]. Briefly, trypsinized cells were incubated in complete medium (phenol red-free Richter's medium with 10% FCS, penicillin and streptomycin) with 10 μM pheophorbide a, 200 nM BODIPY-prazosin or 20 μM mitoxantrone in the presence or absence of 10 μM of the ABCG2 inhibitor FTC for 30 min at 37°C in 5% CO_2. When determining whether becatecarin could be used as a fluorescent substrate for detection of ABCG2 expression, it was used at a concentration of 10 μM. Cells were then washed and allowed to incubate in substrate-free medium continuing with or without FTC for 1 h to generate the FTC/Efflux and Efflux histograms, respectively. The difference in fluorescence (measured in mean channel numbers) between the Efflux and FTC/Efflux histograms was termed FTC-inhibitable efflux and was found in earlier studies to be proportional to ABCG2 expression [20, 21]. When measuring cell surface expression of ABCG2 with

the monoclonal antibody 5D3, trypsinized cells were incubated with phycoerythrin-labeled 5D3 (eBioscience, San Diego, CA, USA) or phycoerythrin-labeled negative control antibody in blocking medium (2% BSA in PBS) according to manufacturer's instructions for 30 min, after which the samples were washed with PBS and analyzed on a flow cytometer.

To determine if becatecarin had an effect on the binding of the 5D3 antibody to ABCG2, we used the 5D3 shift assay first proposed by Ozvegy-Laczka et al. [22] with minor modifications. ABCG2-transfected cells were preincubated in blocking medium containing various concentrations of becatecarin or 10 μM FTC for 30 min at 37°C, after which unlabeled 5D3 antibody (eBioscience, San Diego, CA, USA) at a 1:5,000 dilution was added and cells were incubated for an additional 1 h. Cells were then washed, resuspended in blocking medium, and incubated with allophycocyanin-labeled secondary antibody (1:100, Leinco Technologies Inc., St Louis, MO, USA). Cells were then washed with PBS and analyzed.

Samples were read on a FACSort Flow Cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser and a 635 nm red diode laser. For all samples, 10,000 events were collected and debris was eliminated by gating on forward versus side scatter. Dead cells were eliminated based on propidium iodide staining.

Cytotoxicity assays

Four-day cytotoxicity assays were performed based on the sulforhodamine B assay reported by Skehan et al. [23]. Cells were plated in 96-well flat bottom plates at a density of 10,000 cells/well (HEK293 lines) or 1,000 cells/well (A549 lines) and allowed to attach overnight at 37°C in 5% CO_2. Chemotherapy agents were added at varying concentrations and the plates were incubated for 96 h in 5% CO_2 at 37°C. Cells were then fixed in 50% trichloroacetic acid, washed and dried. Subsequently, the plates were stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid), washed, and dried before sulforhodamine B was solubilized in Trizma base. Optical densities were then determined at an absorbance of 540 nm. Drug concentrations were tested in quadruplicate, and controls were performed in replicates of eight.

[^{125}I]-IAAP labeling of ABCG2

Crude membranes from MCF-7 FLV1000 cells, isolated as previously described [24], were incubated with 20 μM becatecarin or FTC in 50 mM Tris–HCl, pH 7.5 to which 3–6 nM [^{125}I]-IAAP (2,200 Ci/mmole) 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 crosslink the radioactive IAAP to ABCG2. Labeled ABCG2 was then immunoprecipitated using 10 μg of BXP-21 antibody (Kamiya Biomedical, Seattle, WA, USA) as described previously [25]. The incorporation of [^{125}I]-IAAP into the ABCG2 band was quantified using a STORM 860 phosphor imager system (Molecular Dynamics, Sunnyvale, CA, USA) with ImageQuaNT software.

ATPase assay

The BeFx-sensitive ATPase activity of ABCG2 was determined as previously reported [26] with minor modifications. Crude membranes isolated from ABCG2-expressing high five insect cells (100 μg/ml protein) were incubated with varying concentrations of becatecarin in the presence or absence of 0.2 mM beryllium sulfate and 2.5 mM sodium fluoride (BeFX) in ATPase assay buffer (0.05 mM KCl, 5 mM sodium azide, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 50 mM MOPS pH 7.5) for 5 min at 37°C. The reaction was started by the addition of 5 mM ATP and was terminated by the addition of 0.1 ml of 5% SDS solution. The amount of inorganic phosphate released was subsequently determined [26].

Results

ABCG2 expression confers marginal resistance to becatecarin

To determine if ABCG2 expression would confer resistance to becatecarin, we performed 4-day cytotoxicity assays on empty vector and wild-type *ABCG2*-transfected HEK-293 cells. The ABCG2-transfected cells were approximately threefold resistant to becatecarin, as shown in Table 1, suggesting that the drug was a relatively weak substrate for ABCG2, compared to the 15-fold resistance observed for topotecan in these cells.

During the course of our study, we found becatecarin to be fluorescent at high concentrations (10 μM) and detectable by flow cytometry (FL-1; excitation 488 nm, emission 520 nm). This led us to study the effects of ABCG2 expression on intracellular becatecarin fluorescence when cells were incubated with the drug. SF295 MX500 and MCF-7 MX100 cells are known to express high levels of ABCG2 [16, 18]. As shown in column 1 of Fig. 1, when these cells were incubated in the presence of 10 μM PhA alone (solid line), lower intracellular PhA fluorescence is observed compared to cells incubated with PhA in the presence of 10 μM of the ABCG2 inhibitor FTC due to transport by ABCG2 (dashed line). Similarly, as observed in the second column of Fig. 1, when SF295 MX500 and MCF-7 MX100

Table 1 Cross-resistance profile of *ABCG2*-transfected HEK 293 cells and A549 Bec150 cells

Drug	Cell line		RR
	pcDNA3.1	R-2	
Becatecarin	0.033 ± 0.013	0.097 ± 0.051*	2.9
Topotecan	0.023 ± 0.013	0.35 ± 0.050*	15
	A549	A549 Bec150	
Becatecarin	0.016 ± 0.0081	0.050 ± 0.022*	3.1
Becatecarin + 5 μM FTC	0.016 ± 0.0085	0.011 ± 0.0063	0.69
Topotecan	0.19 ± 0.11	1.3 ± 0.77*	6.8
SN-38	0.042 ± 0.019	0.33 ± 0.28*	8.0
Mitoxantrone	0.019 ± 0.0035	0.29 ± 0.011*	15

IC_{50} values are in μM and were obtained using the sulforhodamine B assay as described in the materials and methods. Results are from at least three independent experiments. Values marked with a * were found to be statistically significantly different from the corresponding parental line ($P < 0.03$)

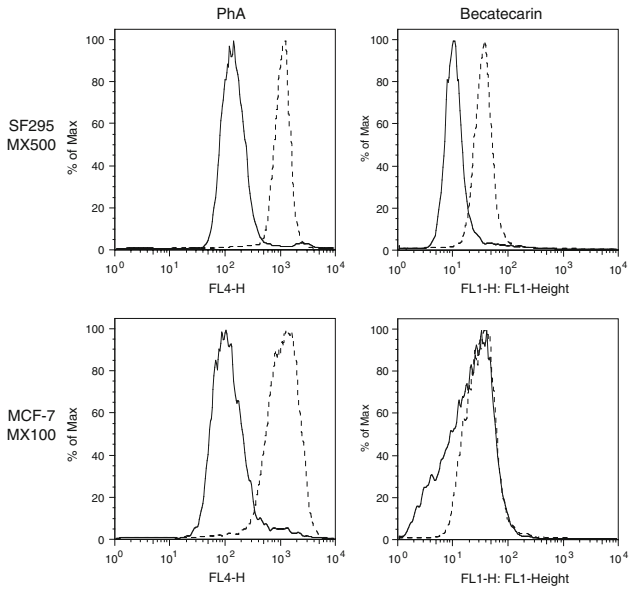

Fig. 1 Becatecarin is transported by ABCG2. MCF-7 MX100 and SF295 MX500 cells were incubated in 10 μM becatecarin in the presence or absence of 10 μM FTC for 30 min, washed, then allowed to incubate and additional 60 min continuing with (*dashed line*) or without (*solid line*) FTC. Results from one of two independent experiments are shown

cells were incubated in 10 μM becatecarin alone (solid line), lower intracellular levels of becatecarin were noted relative to levels observed in cells incubated with becatecarin in the presence of 10 μM FTC (dashed line). The separation of peaks for the MCF-7 MX100 cell line, however, was not as clear as for the SF295 MX500 line. This result confirmed cytotoxicity assay results and suggested becatecarin is an ABCG2 substrate. A range of drug-selected cells were examined for their ability to transport becatecarin, but transport of becatecarin was only noted in cells expressing high levels of ABCG2 (data not shown).

Becatecarin stimulates ATPase activity and competes [^{125}I]-IAAP labeling of ABCG2

Some substrates of ABC transporters are known to stimulate ATPase activity, and it has been shown that ABCG2 substrates, such as mitoxantrone, increase ATPase activity of the protein while inhibitors such as Ko143 decrease ATPase activity [27], although this is not always so clearly delineated. The effect of becatecarin on the ATPase activity of ABCG2 is shown in Fig. 2a. Becatecarin stimulated ATPase activlty in a dose-dependent manner with an apparent K_d of 0.28 μM, and the maximum stimulation was about 1.9-fold. These data were in agreement with the cytotoxicity data suggesting that becatecarin is a substrate of ABCG2.

To confirm the interaction of becatecarin with ABCG2, we next examined the ability of becatecarin to inhibit [^{125}I]-IAAP labeling of ABCG2. Previous reports have shown that both substrates and inhibitors of ABCG2 can prevent labeling of ABCG2 by [^{125}I]-IAAP [25, 28]. Becatecarin inhibited labeling of ABCG2 by [^{125}I]-IAAP in a dose-dependent manner with an IC_{50} of 2.1 μM (Fig. 2b), thus suggesting that becatecarin likely interacts with ABCG2 at the IAAP binding site.

Becatecarin acts as an ABCG2 inhibitor at high concentrations

It has been shown previously that, at high concentrations, ABCG2 substrates such as flavopiridol can act as inhibitors of ABCG2 [22]. Thus, the ability of becatecarin to inhibit ABCG2-mediated pheophorbide a transport was determined (Fig. 3, left column). As shown in Fig. 3, when ABCG2-transfected cells were incubated in pheophorbide a alone (solid line), relatively low intracellular fluorescence levels are observed. When the transfected cells were incubated in pheophorbide a in the presence of 10 μM FTC (dashed line), intracellular pheophorbide a fluorescence was increased (top histogram). Becatecarin was found to not be fluorescent at the same excitation and emission wavelengths (FL-4) used to detect pheophorbide a (data not shown). When ABCG2-transfected cells were incubated in increasing levels of becatecarin, a slight increase in intracellular pheophorbide a fluorescence is noted at 1 μM becatecarin, with 10 μM and 25 μM becatecarin having an effect nearly equal to that of 10 μM FTC. These results suggested becatecarin could act as an inhibitor of ABCG2.

Building on these results, the ability of becatecarin to increase binding of the anti-ABCG2 antibody 5D3 was sub-

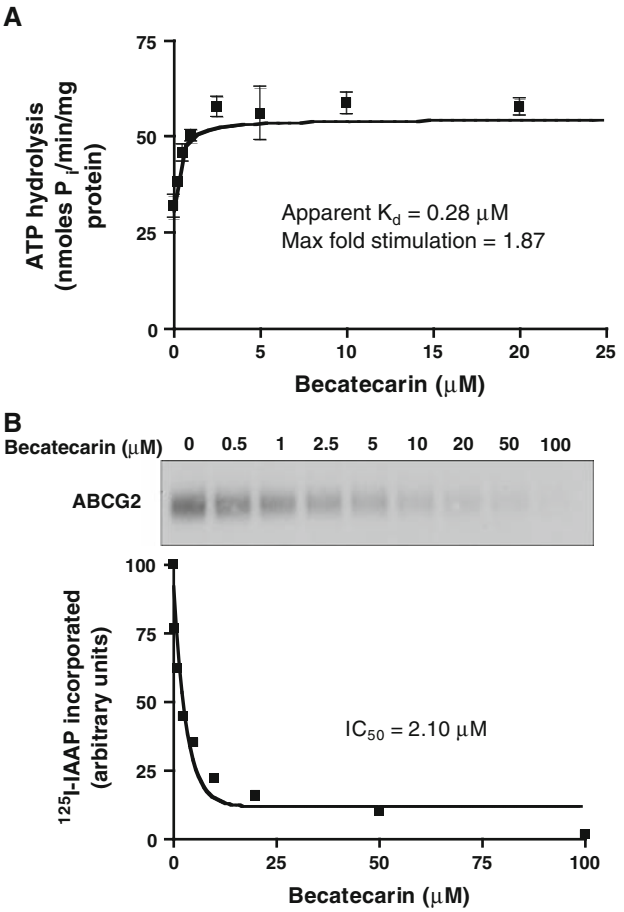

Fig. 2 Becatecarin increases ATPase activity of ABCG2 and competes [^{125}I]-IAAP labeling. **a** The BeFx-sensitive ATPase activity of ABCG2 was determined in the presence of various concentrations of becatecarin as outlined in the materials and methods. **b** Crude membranes isolated from MCF-7 FLV1000 cells were incubated with various concentrations of becatecarin in the presence of [^{125}I]-IAAP followed by UV crosslinking. The radioactivity incorporated into the band was quantitated as described in the "Materials and methods" section. Results from one of two independent experiments are shown

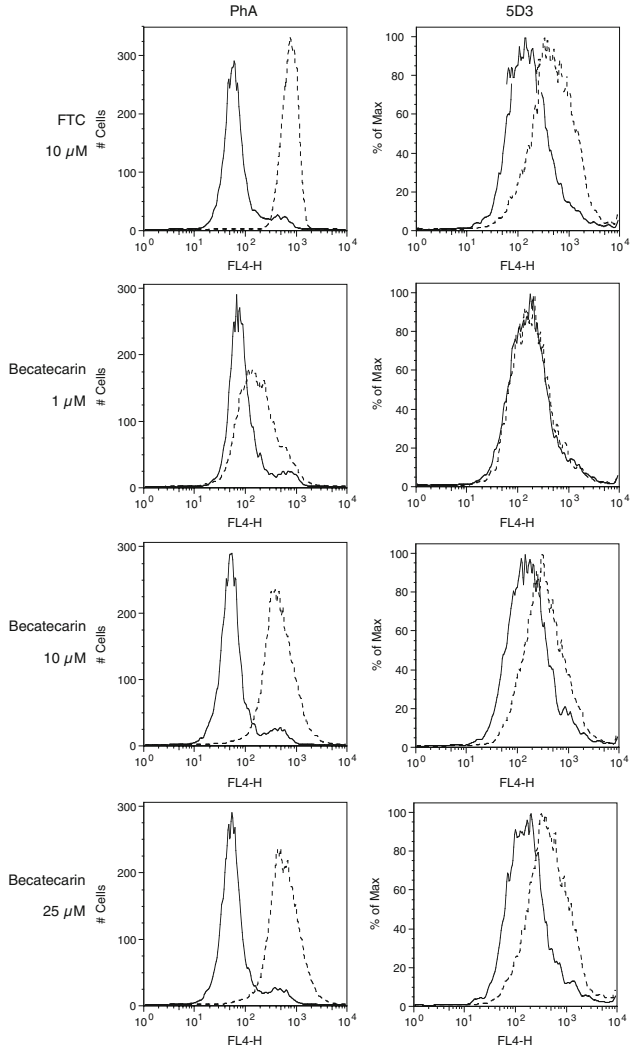

Fig. 3 Inhibition of ABCG2-mediated transport by becatecarin. *Left column* ABCG2-transfected cells were incubated in 1 μM PhA in the presence or absence of the desired concentration of becatecarin or 10 μM FTC for 30 min, washed, then allowed to incubate for 60 min continuing with (*dashed line*) or without (*solid line*) inhibitor. *Right column* ABCG2-transfected cells were preincubated for 30 min in the presence (*dashed line*) or absence (*solid line*) of the desired inhibitor after which unlabeled 5D3 antibody (1:5,000) was added and incubated for an additional 30 min. Cells were then washed and incubated with APC-conjugated secondary antibody. Results from one of two independent experiments are shown

sequently examined (Fig. 3, right column). Ozvegy-Laczka and colleagues have previously shown that ATPase inhibitors or compounds that act as ABCG2 inhibitors increase binding of the 5D3 antibody to ABCG2 at high dilutions of 5D3 [22]. We have confirmed these results, noting that this shift is observed only when very low levels of antibody are used (data not shown). When ABCG2-transfected cells were incubated with 10 or 25 μM becatecarin in the presence of the 5D3 antibody (1:2,500 dilution, dashed line), an increase in fluorescence is noted compared to cells incubated with 5D3 antibody alone (solid line), confirming the ability of becatecarin to act as an inhibitor at high concentrations. This effect was comparable to that of 10 μM FTC which is shown as a positive control (Fig. 3, right column, top histogram).

Characterization of becatecarin-resistant A549 lung carcinoma cells

Becatecarin-resistant cells were then developed to evaluate whether ABCG2 might emerge as a mechanism of resistance in response to selection with the drug. A549 non-small cell lung cancer cells were chosen for this study since ABCG2 is expressed endogenously in this cell line. A549 cells were selected in increasing amounts of becatecarin, resulting in the A549 Bec150 subline which is

maintained in 150 nM becatecarin. Cytotoxicity assays revealed that the Bec150 subline is 3.1-fold resistant to becatecarin, but also 15-fold resistant to mitoxantrone, 6.8-fold resistant to topotecan and 8-fold resistant to SN-38. The IC_{50} values for the resistant line were significantly different from the parent line with $P < 0.03$ for all drugs tested. Since this pattern of cross-resistance suggested that the cells expressed ABCG2, we performed cytotoxicity assays with becatecarin in the presence or absence of 5 μM of the ABCG2 inhibitor FTC. As shown in Table 1, the addition of FTC completely reversed resistance to becatecarin in the A549 Bec150 line, while the IC_{50} for becatecarin in the parent line was unchanged with the addition of FTC.

To confirm overexpression of ABCG2 in the Bec150 subline, flow cytometry-based studies with the 5D3 antibody at concentrations that are not affected by ABCG2 conformation, and the fluorescent ABCG2 substrates pheophorbide a, mitoxantrone, and BODIPY-prazosin were performed. As seen in Fig. 4, when A549 parental and Bec150 cells were incubated with phycoerythrin-labeled 5D3 antibody (dashed line) or an isotype control (solid line) at low dilutions (1:20), low levels of ABCG2 expression were found in the parental cells, as shown by the slight difference between the solid and dashed lines (first histogram, top row). In the Bec150 line, elevated levels of ABCG2 were detected, shown by the greater difference between the solid and dashed lines (second histogram, top row).

Increased transport of the ABCG2 substrates pheophorbide a, mitoxantrone, and BODIPY-prazosin was also observed in the drug-selected subline compared to parental A549 cells. A549 cells were previously shown to have low but detectable levels of functional ABCG2 activity as evidenced by the small amount of FTC-inhibitable efflux of pheophorbide a, mitoxantrone and BODIPY-prazosin (small difference between solid and dashed histograms in second, third and fourth histograms in first column of Fig. 4). In contrast, A549 Bec150 cells showed markedly higher levels of ABCG2 transporter activity, demonstrated by the increased difference between the solid and dashed histograms.

Immunoblot studies were also undertaken to determine if other ABC transporters associated with drug resistance could be detected in the drug-selected line. Microsomal membrane protein was isolated from A549 and Bec150 cells as well as HEK-293 cells transfected with *ABCB1*, *ABCC1*, and *ABCG2* that served as positive controls for Pgp, MRP1 and ABCG2 expression, respectively. As seen in Fig. 5, A549 Bec150 cells were found to overexpress ABCG2, but not Pgp or MRP1, suggesting that resistance to becatecarin in the A549 Bec150 line is due primarily to ABCG2 expression.

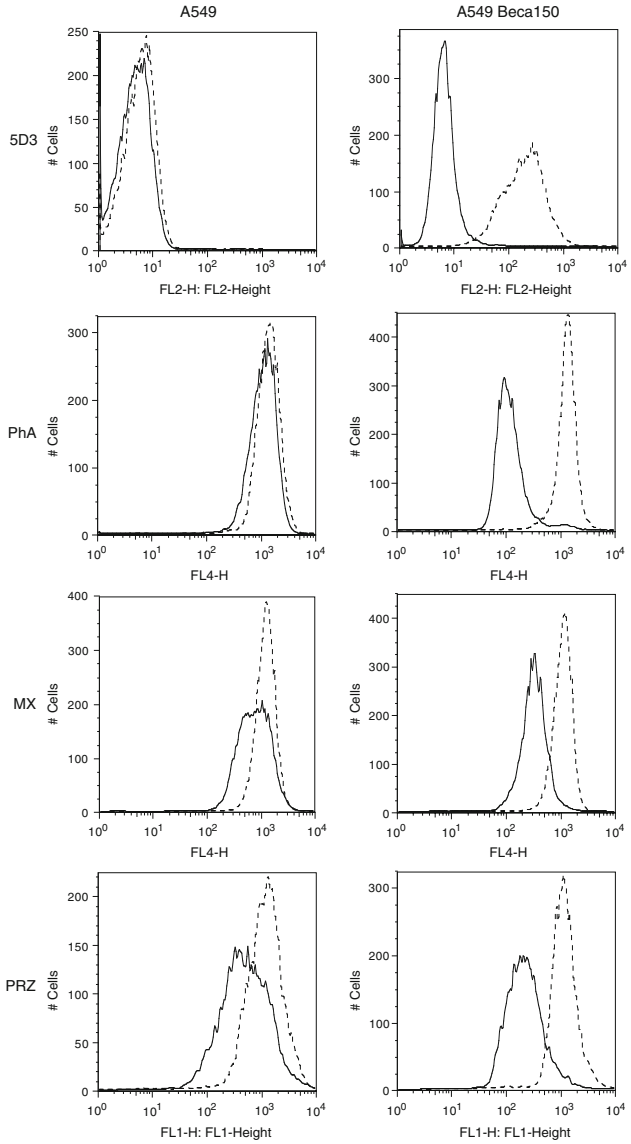

Fig. 4 Increased ABCG2 expression in becatecarin-selected A549 cells as measured by flow cytometry. A549 parental (*left column*) or A549 Bec150 (*right column*) cells were incubated in the presence of phycoerythrin-labeled 5D3 anti-ABCG2 monoclonal antibody (*dashed line*) or phycoerythrin-labeled IgG2b negative control antibody (*solid line*) for 30 min after which cells were washed and read on a flow cytometer (*top row*). Cells were incubated in 10 μM PhA (PhA, *second row*), 20 μM mitoxantrone (MX, *third row*), or 200 nM BODIPY-prazosin (PRZ, *bottom row*) in the presence or absence of 10 μM FTC for 30 min, washed, and subsequently incubated in substrate-free medium for 1 h continuing with (*dashed line*) or without (*solid line*) FTC

Discussion

In the present study, we demonstrate that becatecarin is a substrate for the ATP binding-cassette half-transporter ABCG2, and that resistance to becatecarin can be reversed by inhibitors of ABCG2 such as FTC. Becatecarin was also

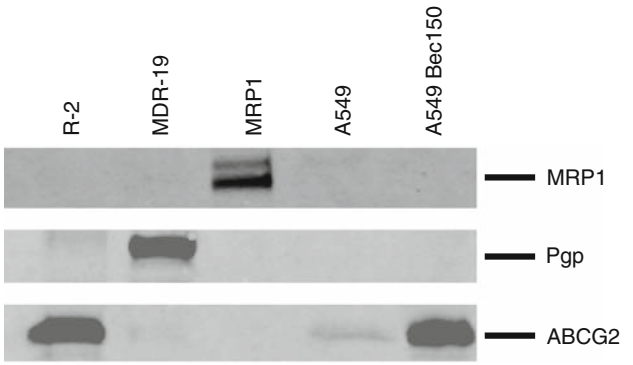

Fig. 5 ABCG2 expression, but not Pgp or MRP1 expression, is upregulated in becatecarin-resistant A549 lung carcinoma cells. Microsomal membrane fractions were separated by SDS-PAGE and transferred to a PVDF membrane that was sequentially probed for Pgp, MRP1 and ABCG2 expression as outlined in the materials and methods. Membrane protein isolated from HEK-293 cells transfected with *ABCB1*, *ABCC1* or *ABCG2* (denoted by R-2, MDR-19 and MRP1, respectively) served as positive controls for Pgp, MRP1 and ABCG2

found to be fluorescent and FTC-inhibitable becatecarin transport was observed in cell lines by flow cytometry. However, becatecarin transport was only observed in drug-resistant cells expressing very high levels of ABCG2, rendering becatecarin a rather insensitive probe for measuring ABCG2 expression. Additionally, we describe a becatecarin-resistant cell line derived from A549 lung carcinoma cells, A549 Bec150, which was found to overexpress ABCG2 as the primary mechanism of resistance.

While becatecarin was found to be transported by ABCG2, it does not appear to be as well transported as other ABCG2 substrates such as mitoxantrone, topotecan or SN-38. This is evidenced by the fact that ABCG2-transfected cells are only about 3-fold resistant to becatecarin, while they were found to be 15-fold resistant to topotecan. Additionally, the becatecarin resistant line was found to be approximately 3-fold resistant to becatecarin, but 7- to 15-fold resistant to MX, topotecan, and SN-38. This is in contrast to studies conducted with the structurally related indolocarbazole compounds compound A (NB-506) [12, 29] and J-107088 [12]. Komatani et al. [12] generated NB-506-resistant HCT-116 cell lines that were 2400-fold resistant to NB-506 and 230-fold resistant to J-107088 but were only 10-fold resistant to mitoxantrone and 4-fold resistant to topotecan. Similarly, Nakagawa et al. found that wild-type ABCG2-transfected MCF-7 cells were 298-fold resistant to NB-506, but only 9-fold resistant to mitoxantrone and 7-fold resistant to topotecan. The rebeccamycin derivative becatecarin is also an indolocarbazole, but differs from NB-506 and J-107088 by the groups at positions 1, 2, 6, 10, 11 and 14 of the indolocarbazole structure [30]. Thus, modifications at these positions may affect recognition by the ABCG2 protein, but further experiments are needed to confirm this theory.

In the present work we demonstrate that continued treatment with becatecarin is able to induce expression of ABCG2 in lung cancer cells despite the fact that becatecarin does not seem to be readily transported by ABCG2. It is possible that becatecarin may induce ABCG2 expression in other types of cancers as well. If such an induction were to occur in patients and becatecarin were indeed able to induce ABCG2 in tumors, there is concern that this would limit the efficacy of subsequent treatments with drugs that were substrates of ABCG2 such as topotecan, irinotecan or mitoxantrone.

A search for single nucleotide polymorphisms (SNPs) in the *ABCG2* gene resulted in the discovery of the C421A polymorphism that results in a glutamine to lysine (Q141 K) change in the protein. Several groups have found that cells expressing Q141 K ABCG2 displayed altered protein expression as well as decreased ATPase activity and altered protein function leading to decreased transport of substrate compounds [31–33]. This SNP was also found to have clinical impact as patients who were homozygous or heterozygous for the SNP were found to have higher plasma concentrations of the intravenously-administered camptothecin analog diflomotecan [34] compared to patients who expressed only wild-type alleles. The effects of the Q141 K SNP were observed with diflomotecan even though *ABCG2*-transfected cells were found to be only threefold resistant to diflomotecan [35]. Since we found becatecarin to be a weak substrate of ABCG2 as well, the Q141 K SNP might also affect disposition of the drug or its metabolites.

One strategy that has been posited in order to overcome drug resistance is to develop compounds that are not substrates for Pgp or MRP1. Unfortunately, compounds that have been developed with the stated advantage of circumventing resistance mediated by Pgp or MRP1 have been observed to be transported by ABCG2. This is true in the case of flavopiridol [17], and also appears true for becatecarin. Even novel targeted therapeutic drugs such as imatinib and nilotinib appear to be substrates, albeit weak ones, for ABC transporters [36, 37]. Thus, our report highlights the fact that ABC transporters continue to remain an important consideration in the development of antineoplastic agents.

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