Drug Efflux by Breast Cancer Resistance Protein Is a Mechanism of Resistance to the Benzimidazole Insulin-Like Growth Factor Receptor/Insulin Receptor Inhibitor, BMS-536924

Xiaonan Hou1, Fei Huang2, Joan M. Carboni2, Karen Flatten1, Yan W. Asmann3, Cynthia Ten Eyck1, Takeo Nakanishi4, Jennifer D. Tibodeau1, Douglas D. Ross4, Marco M. Gottardis2, Charles Erlichman1, Scott H. Kaufmann1, and Paul Haluska1

Abstract

Preclinical investigations have identified insulin-like growth factor (IGF) signaling as a key mechanism for cancer growth and resistance to clinically useful therapies in multiple tumor types including breast cancer. Thus, agents targeting and blocking IGF signaling have promise in the treatment of solid tumors. To identify possible mechanisms of resistance to blocking the IGF pathway, we generated a cell line that was resistant to the IGF-1R/InsR benzimidazole inhibitors, BMS-554417 and BMS-536924, and compared expression profiles of the parental and resistant cells lines using Affymetrix GeneChip Human Genome U133 arrays. Compared with MCF-7 cells, breast cancer resistance protein (BCRP) expression was increased 9-fold in MCF-7R4, which was confirmed by immunoblotting and was highly statistically significant (P = 7.13E-09). BCRP was also upregulated in an independently derived resistant cell line, MCF-7 924R. MCF-7R4 cells had significantly lower intracellular accumulation of BMS-536924 compared with MCF-7 cells. Expression of BCRP in MCF-7 cells was sufficient to reduce sensitivity to BMS-536924. Furthermore, knockdown of BCRP in MCF-7R4 cells resensitized cells to BMS-536924. Four cell lines selected for resistance to the pyrrolotriazine IGF-1R/InsR inhibitor, BMS-754807, did not have upregulation of BCRP. These data suggest that benzimidazole IGF-1R/InsR inhibitors may select for upregulation and be effluxed by the ATP-binding cassette transporter, BCRP, contributing to resistance. However, pyrrolotriazine IGF-1R/InsR inhibitors do not appear to be affected by this resistance mechanism. Mol Cancer Ther; 10(1); 117–25. ©2011 AACR.

Introduction

The insulin-like growth factor (IGF) pathway is a key system that contributes to proliferation and prosurvival signaling in a wide variety of tumor types (1, 2) It also has been implicated in the resistance to many useful anticancer agents (3, 4). As such, there is great interest in the development of therapeutic antagonists of the IGF system. Currently, several inhibitors of the IGF system are in preclinical and clinical development (5–7). Most of these agents target the IGF signaling receptors, IGF-1R and the insulin receptor (InsR). As these agents move forward in clinic, it will be important to identify determinants of sensitivity to targeting the IGF-1R pathway (8).

Among the most important determinants of resistance to anticancer therapies are the ATP-binding cassette (ABC) family of transporters that are responsible for drug efflux of structurally diverse toxins, dyes, and other xenobiotics (9–11). In cancer therapy, enhanced expression of ABC transporters has been implicated in the resistance to a number of clinically important chemotherapeutics (12–14). Among the most extensively characterized is P-glycoprotein (P-gp, MDR, ABCB1), which has been implicated as a major mechanism of resistance to paclitaxel, doxorubicin, and vincristine (15, 16). Another transporter, ABCG2 [breast cancer resistance protein (BCRP)], has been implicated in resistance to camptothecin analogues and mitoxantrone (17, 18).

Upon trying to understand the mechanisms of resistance to small molecule, tyrosine kinase inhibitors of IGF-1R, we found increased expression of BCRP in cells resistant to the benzimidazole IGF-1R/InsR inhibitors, BMS-554417 and BMS-536924 (Fig 1A; refs. 19, 20). We now report that BCRP is able to efflux the BMS-536924, reducing its intracellular concentration and is sufficient to confer resistance. However, upregulation of BCRP was

Authors' Affiliations: 1Department of Oncology and 2Division of Biomedical Informatics, Mayo Clinic, Rochester, Minnesota; 3Oncology Drug Discovery, Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey; and 4University of Maryland Greenebaum Cancer Center and Baltimore Veterans Medical Center, Baltimore, Maryland

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Current address for J.D. Tibodeau: R&D Chemistry Assay Development, Beckman Coulter, Chaska, MN 55318.

Corresponding Author: Dr. Paul Haluska, Division of Medical Oncology, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905. Phone: 507-266-0029; Fax: 507-284-1803. Email: haluska.paul@mayo.edu
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not seen in a diverse panel of cell lines resistant to the pyrrolotriazine IGF-1R/InsR inhibitor, BMS-754807 (Fig. 1A; refs. 21, 22). These data suggest that BCRP status may be important in predicting tumor response to the benzimidazole class of compounds.

Materials and Methods

Reagents were obtained from the following suppliers: bovine serum albumin, ampicillin, DAPI, Hoechst 33258, SDS, bromphenol blue, and glycerol from Sigma; SDS-PAGE reagents from BioRad; FBS, PBS, pcDNA 3.0 mammalian expression vector, Stealth RNAi against ABCG2 (HSS114013), Stealth RNAi Low GC negative control (12935-200), Lipofectamine RNAiMax, Opti-MEM, and trypsin-EDTA from Gibco/Invitrogen; chicken and goat polyclonal antibodies against actin from Abcam; DMEM medium, sodium pyruvate, and penicillin/streptomycin from Cellgro/MediaTech; CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit from Promega; and BCRP monoclonal antibody, clone BXP-21 from Calbiochem. MCF-7 (breast cancer) and SW403 (colon cancer) cell lines were obtained from American Type Culture Collection (ATCC). Geo (colon cancer) cell line was a kind gift from K. Mulder (Penn State College of Medicine, Hershey, PA). Rh1 (Ewing sarcoma) and Rh41 (rhabdomyosarcoma) cell lines were kind gifts from L. Helman (National Cancer Institute). The mouse monoclonal antibody against lamin A and C was a kind gift from F. McKeon (Harvard Medical School, Boston, MA). The antibody recognizing BCRP in Fig. 4A was a kind gift from G. Scheffer (Free University Hospital, Amsterdam, The Netherlands).

Cell culture and cell line construction.

MCF-7 cells were obtained and grown as previously described (19). MCF-7R4 cells were generated by stepwise growth in increasing concentrations of BMS-554417 over the course of approximately 9 months. At a concentration of 10 μmol/L, ~40-fold greater than the determined IC<sub>50</sub> of the parental cells, individual clones were selected by disbursement of cells to generate colonies from single cells. Colonies were then selected using cloning rings and expanded in cell culture medium in the presence of 10 μmol/L of BMS-554417. MCF-7R4 represented a clone that proliferated under continued selection with BMS-554417, a first-generation IGF-1R/InsR inhibitor at 10 μmol/L and is cross-resistant to the IGF-1R/InsR inhibitor BMS-536924, a structurally related analogue of BMS-554417. MCF-7 924R was selected in a similar fashion with BMS-536924 and MCF-7/BCRP stable transfectants were generated by electroporating parental MCF-7 cells with pcDNA3.0 encoding wild-type BCRP at 240 V for 10 ms using a BTX820 square wave electroporator, selecting for stable transfectants in 800 μg/mL of geneticin, isolating individual clones using cloning rings, and screening for expression by immunoblotting with anti-BCRP antibody. Empty vector controls were generated similarly using the pcDNA 3.0 vector.

All cell lines from either ATCC or as gifts were expanded and frozen into multiple aliquots. The resultant aliquots used for experiments originated from the source aliquots and were passaged for up to 6 months. The exceptions were cell lines used for acquired resistance. All lines used for the resistant cell line panel were authenticated by SNP 6.0 array analysis (Affymetrix). No additional authentication was performed on the BCRP and pcDNA vector transformed variants of MCF-7 cells. Cell lines were either purchased or acquired as gifts within the last 5 years.
Proliferation assay

MTS assays were performed as described previously with modifications (23). Briefly, subconfluent cells were seeded into 96-well plates at 5,000 cells per well. After incubation in serum-containing medium overnight, the medium was switched to serum-free conditions in the absence or presence of BMS-536924 at varying concentrations. After 72 hours of growth, viable cell mass was assessed by MTS dye reduction.

Clonogenic assays

Clonogenic assays were performed as previously described (23). Briefly, MCF-7 cells and variants, including MCF-7R4, RNA-treated MCF-7R4 (Fig 4C), MCF-7 BCRP and vector control stable transfectants (Fig 4A), were trypsinized and plated in 60-mm tissue culture plates to a density of 500 to 1,000 per plate, respectively. Cells were allowed to adhere for 22 to 24 hours, and drugs were then added as indicated to final concentrations from 1,000-fold concentrated stocks. After 72-hour incubation, plates were washed and incubated in fresh serum-containing medium until colonies were visible. Colonies were counted and reported as percent of control (DMSO treated) cells. Experiments were performed in triplicate. Differences among treatment groups were assessed statistically by 2-way ANOVA and IC50 values were calculated by a nonlinear normalized response curve using Prism 5.0 (GraphPad Software).

Western blot analysis

Cellular lysates were assayed by immunoblotting as previously described (23). Briefly, subconfluent cells, under conditions described in the text, were washed twice with ice-cold PBS and lysed on the plates or flasks with 4× sample buffer (4×SB). The lysate was then sonicated and equal quantities of protein were resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) or nitrocellulose and probed with the indicated antibodies as per the vendor’s recommendations.

Microarray analysis

Five replicates of total RNA were isolated from MCF-7 and MCF-7R4 cells using RNeasy Midi kit (Qiagen). Microarray analysis was conducted according to manufacturer’s instructions for the Affymetrix One Cycle Target Labeling and Control Reagents kit. Briefly, 5 μg of total RNA was used to synthesize double-stranded cDNA using SuperScript II reverse transcriptase (Invitrogen) and T7 Oligo (dT) primer. Subsequently, the products were column purified (Affymetrix) and then in vitro transcribed to generate biotin-labeled cRNA. The IVT products were then column purified, fragmented, and hybridized to Affymetrix GeneChips (U133A or U133 Plus 2.0, as indicated in Results) at 45°C for 16 hours. Subsequent to hybridization, the arrays were washed and stained with streptavidin-phycocerythrin, then scanned in an Affymetrix GeneChip Scanner 3000. All control parameters were confirmed to be within normal ranges before normalization and data reduction was initiated. The digitalized raw image (CEL) files were read, normalized, and gene expression calculations were performed using dChip (http://biosun1.harvard.edu/~cli/dchip2006.exe) PM-only and invariant set normalization, and polished median summarization of probe sets. All absent genes were excluded from further analysis.

Intracellular BMS-536924 concentration determinations

Cells treated with BMS-536924 at various concentrations (0.5, 1, 5, and 10 μmol/L) for 1 hour at 37°C were harvested with trypsin containing BMS-536924 at the same concentrations (0.5, 1, 5, and 10 μmol/L). Cells were then counted and pelleted in ice-cold PBS. BMS-536924 was extracted by lysing the cell pellets in 100 μL of 10% TCA. Aliquots were injected onto a Beckman System Gold HPLC system and separated on a Beckman Ultrasphere ODS 5 μmol/L of column (4 mm x 25 cm) and eluted using a 1:1 Acetonitrile; 2.5 mmol/L of ammonium formate (pH 4.0) mobile phase. Eluted BMS-536924 was quantitated in arbitrary units against the standards using a UV absorbance detector set to monitor at 350 nm. Stock BMS-536924 in 10% TCA was used as standard. Samples were corrected for cell numbers and intracellular concentration was reported as arbitrary absorbance units per cell, based on cell volume as previously described (24). Experiments were performed in triplicate.

Fluorescence microscopy

To visualize uptake of the BMS-536924, live, adherent cells treated as described in the text, were imaged qualitatively using a DAPI filter set as previously described (23).

BCRP knockdown

In 6-well plates, 500 μL of Opti-MEM was added per well in combination with 100 pmol of either scrambled RNA or BCRP targeting RNAi (HSS114013, sense sequence GGGGACCTTTGAATCAGCTGGTTAT) and 30 μL of Lipofectamine RNAiMax and rocked at room temperature for 20 minutes. A total of 2.5 × 105 cells in 2.5 mL of Opti-MEM were then added per well and incubated for 96 hours. Cells are then trypsinized and subjected to clonogenic assays or Western blotting as described above.

Results

ABCG2 is highly upregulated in MCF-7R4 cells

Compared with the parental MCF-7 breast cancer cells, MCF-7R4 cells are approximately 40-fold resistant to the IGF-1R inhibitor, BMS-554417 (Fig. 1B) and are cross-resistant to BMS-536924 (Fig. 1C; ref. 20). To investigate differences in the expression profile of breast cancer cells resistant and sensitive to IGF-1R inhibition, we performed a DNA microarray experiment on MCF-7R4
and MCF-7 cells. On comparing the genes with the greatest difference in expression, we identified ABCG2 (Fig. 2A). As a known member of a family of drug efflux pumps, we hypothesized that ABCG2 represented a rational mechanism to confer resistance to BMS-536924. Accordingly, it was selected for further evaluation.

**Upr egulation of ABCG2 is specific among ABC transporters**

To show that global ABC transporter expression was not upregulated in MCF-7R4 cells and that this was specific to ABCG2, we compared the expression profile differences for all ABC transporters represented on the Affymetrix GeneChip U133 Plus 2.0 platform (Fig. 2B and Supplementary Fig. S1). Although ABCG2 was upregulated nearly 9-fold compared with the parental MCF-7 cells, all other detected ABC transporter transcripts showed less than a 3-fold upregulation (Fig. 2B and Supplementary Fig. S1). Most, including the transcript for MDR1/ABCB1, were not significantly changed, suggesting this upregulation was highly specific to ABCG2. MCF-7 924R, a separately isolated clone developed in increasing concentrations of BMS-536924, also had ABCG2 upregulation as the largest change in ABC transporter expression compared with parental cells (Supplementary Fig. S2A and B).

**BCRP protein is highly upregulated in MCF-7R4 cells**

To verify that the upregulation of ABCG2 mRNA resulted in increased expression of the gene product, BCRP, we performed Western blot analysis (Fig. 2C). Compared to the parental MCF-7 cells, BCRP was greatly increased in MCF-7R4 cells. P-gp, the gene product of MDR1/ABCB1 was not detectable in MCF-7R4 or MCF-7 (data not shown).

**Accumulation of BMS-536924 is altered in MCF-7R4**

Having shown that MCF-7R4 cells express high levels of BCRP, we investigated whether this expression had a functional effect on the intracellular accumulation of BMS-536924. We observed that BMS-536924 was fluorescent with UV stimulation and visible under a DAPI filter using an excitation wavelength 380 nm and emission wavelength of 430 nm (23). We exploited this finding to assess the cellular accumulation of BMS-536924 in MCF-7 and MCF-7R4 cells. Noticeable qualitative differences in the intensity of the BMS-536924–induced fluorescence were observed in MCF-7 cells compared with MCF-7R4 cells (Fig. 3A). To assess this quantitatively, we compared the intracellular accumulation of BMS-536924 in both MCF-7 and MCF-7R4 cells at various concentrations. Using a high-performance liquid chromatography method for quantitating the concentrations of BMS-536924 extracted from MCF-7 and MCF-7R4 lysates, we determined that MCF-7R4 cells had diminished accumulation compared with parental cells (Fig. 3B).

**MCF-7 cells overexpressing BCRP are resistant to BMS-536924**

To further test the hypothesis that BCRP was responsible for efflux of BMS-536924, MCF-7 cell variants were generated by stable transfection with either the empty vector or full-length wild-type cDNA encoding BCRP. Two clones (B1 and B21) overexpressing BCRP (Fig. 4A) were subjected to further analysis. Both BCRP-expressing clones were significantly less sensitive to BMS-536924 than either the nontransfected MCF-7 cells (P < 0.0001) or MCF-7 cells stably transfected with pcDNA (P < 0.001; Fig. 4B). Expression of BCRP resulted in a 4-fold increase in the IC50 values (B1, 9.6 μmol/L; B21, 10.8 μmol/L), compared with the parental cells (2.4 μmol/L) and 3-fold increase over the empty vector transfectants (IC50, 3.8 μmol/L). These data suggested that BCRP expression is sufficient to decrease sensitivity to BMS-536924.

To further confirm the role of BCRP overexpression in BMS-536924 resistance, we used siRNA to decrease the expression of BCRP in MCF-7R4 cells. Compared with MCF-7R4 cells treated with control scrambled siRNA, siRNA targeting BCRP led to significant downregulation of BCRP and increased BMS-536924 sensitivity (P < 0.001; Fig. 4C and D). The IC50 decreased 3-fold from approximately 17.6 to 4.9 μmol/L. These data suggested that BCRP contributed to high-level resistance to BMS-536924.

**BMS-754807 does not induce ABCG2 upregulation**

Given that the upregulation of BCRP occurred in response to 2 IGF-1R/InsR inhibitors with benzimidazole backbone structures, we sought to determine if this was specific to this class of compounds. It was conceivable that other classes of IGF-1R tyrosine kinase inhibitors would also induce BCRP upregulation. To test this hypothesis, we developed a cell line, MCF-7-807, that was resistant to the pyrrolotriazine inhibitor, BMS-754807, compared with parental MCF-7 cells. BCRP was not upregulated in MCF-7-807 cells compared with control MCF-7 cells (Fig. 5A). In addition to this, we also assessed ABCG2 expression in a panel of 4 cell lines that were developed with acquired resistance to BMS-754807 (21, 22). Using the Affymetrix GeneChip U133A platform, the expression profiles of Geo-807R, SW403-807R, Rh1-807R, and Rh41-807R were compared with the sensitive parental cell lines (Geo, SW403, Rh1, and Rh41). Unlike MCF-7R4 and MCF-7 924R cells, upregulation of ABCG2 was not observed in any of the cell lines selected for resistance to BMS-754807 (Fig. 5B). To further test the hypothesis that sensitivity to pyrrolotriazine IGF-1R inhibitors was independent of BCRP expression, we assessed the proliferation of both parental MCF-7 cells and the MCF-7 BCRP clone B1 stable transfectants in the absence and presence of increasing concentrations of BMS-754807. Both cell lines were similar in sensitivity to BMS-754807 (Fig. 5C).
Figure 2. ABCG2 is upregulated in MCF-7R4 cells. A, heat map representing the most significantly upregulated (top genes) or downregulated (bottom genes) in MCF-7R4 cells compared with MCF-7 parental cells. Each column represents 5 replicates of DNA microarray analyses from either MCF-7 or MCF-7R4 cells. Adjacent to heat map is fold change of gene expression (7R4/7) and P-value for t-test. The arrow indicates ABCG2/BCRP. Complete DNA microarray raw data are submitted to the GEO repository at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18912. B, changes in ABC transporter family members represented in microarray experiments in MCF-7R4 cells compared with parental MCF-7 cells. For clarity, only genes with MCF-7R4/MCF-7 expression ratio less than 1 are shown. *, ABCG1/BCRP; †, ABCB1/P-gp. (Genes with ratio < 1 are shown in Supplementary Fig. S2). Error bars represent SD. C, Western blotting of lysates from MCF-7R4 cells for ABCG1/BCRP versus parental MCF-7 cells. Blots are also probed for actin as a loading control. Blot is representative of 3 experiments.

**Figure 2.**

**A**

- Heat map representing the most significantly upregulated (top genes) or downregulated (bottom genes) in MCF-7R4 cells compared with MCF-7 parental cells. Each column represents 5 replicates of DNA microarray analyses from either MCF-7 or MCF-7R4 cells. Adjacent to heat map is fold change of gene expression (7R4/7) and P-value for t-test. The arrow indicates ABCG2/BCRP. Complete DNA microarray raw data are submitted to the GEO repository at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18912.

**B**

- Changes in ABC transporter family members represented in microarray experiments in MCF-7R4 cells compared with parental MCF-7 cells. For clarity, only genes with MCF-7R4/MCF-7 expression ratio less than 1 are shown. *, ABCG1/BCRP; †, ABCB1/P-gp. (Genes with ratio < 1 are shown in Supplementary Fig. S2). Error bars represent SD.

**C**

- Western blotting of lysates from MCF-7R4 cells for ABCG1/BCRP versus parental MCF-7 cells. Blots are also probed for actin as a loading control. Blot is representative of 3 experiments.
Figure 3. MCF-7R4 cells have altered uptake of BMS-536924 due to increased BCRP expression. A, MCF-7 cells transfected with an empty expression vector (MCF-7/pcDNA3) and visualized by fluorescent microscopy using a DAPI filter set or light microscopy (phase contrast) in the presence (+) or absence (−) of BMS-536924 (924) while growing on culture plates as described in the Materials and Methods section. MCF-7R4 cells and MCF-7 parental cells stably transfected with pcDNA3 containing the full-length wild-type ABCG2 cDNA were also visualized. Images are representative fields from 3 replicate experiments. B, MCF-7 or MCF-7R4 cells growing in culture dishes were exposed to increasing concentrations of BMS-536924. Cells were then harvested and BMS-536924 was extracted, resolved by HPLC and quantified by fluorescence detection against BMS-536924 standards, as described in the Materials and Methods section. Error bars represent SE.
Discussion

We have identified the IGF-1R tyrosine kinase inhibitor BMS-536924 as a newly identified substrate for the ABC transporter, BCRP. In MCF-7R4 cells, BCRP overexpression was selected for by exposure to increasing concentrations of the benzimidazole derivative inhibitor BMS-554417. The upregulation of BCRP in response to IGF-1R inhibition occurred with little change in any other ABC transporter, suggesting that this class of compounds specifically promote the expression of this efflux pump. These data suggest that BCRP expression may be important in patient selection and predicting which patients are less likely to get benefit from this therapy. Immunohistochemical assays for determining BCRP expression status are commercially available and can be assayed on archival tissue.

However, it is unknown if BCRP upregulation would occur in patients taking benzimidazole inhibitors. As the finding was demonstrated in 1 cell line, albeit independently observed in 2 separate laboratories, the frequency of BCRP upregulation in response to benzimidazole inhibitors in breast or other cancers is unknown.

Alternatively, benzimidazole inhibitors such as BMS-536924 could possibly serve as inhibitors of BCRP efflux. The non-anticancer benzimidazole proton pump inhibitors, pantoprazole and omeprazole, effectively inhibit methotrexate efflux by BCRP (25). Additional work will be necessary to show that BMS-536924 is capable of inhibiting efflux of BCRP substrates to improve the activity of chemotherapeutic agents in cancer such as doxorubicin and topotecan. Recently, data have demonstrated that BCRP-mediated drug efflux camptothecin...
analogues can be blocked by small molecule inhibitors of erbB receptors, reversing resistance (18). Interestingly, erbB receptor signaling has been found to be sufficient to overcome sensitivity to IGF-1R inhibition (23, 26). These data have supported the hypothesis of a cross-talk receptor signaling system, whereby proliferative and prosurvival signaling by erbB receptors (HER2 or EGFR) and IGF-1R are redundant and compensatory upon blockade. By preventing cross-talk between the IGF-1R and erbB receptors, as well as blocking IGF-1R/InsR tyrosine kinase inhibitor efflux, there is rationale for combining IGF-1R tyrosine kinase inhibitors with erbB receptor tyrosine kinase inhibitors in clinical investigations.

Although BCRP was responsible for significantly reducing the sensitivity of MCF-7R4 cells to BMS-536924, it is not able to completely reverse resistance in MCF-7R4 to the level of the parent cell line. Thus, it is likely that other alterations in MCF-7R4 cells are contributing to decrease sensitivity to BMS-536924. Of note, changes in the targets IGF-1R and InsR, as well as the basal level of activation was no different in MCF-7 cells in comparison with the parental cells (Supplementary Fig. S3), nor were any mutations in IGF-1R coding region identified (data not shown). On the basis of the DNA microarray data generated in these studies (Fig. 2A), there are additional upregulated candidates that may be important including CXCR4, which may induce a more malignant phenotype; UGT1A10, which may enhance drug elimination; and IGFBP-5, which may reduce IGF ligand availability and alter the cellular responsiveness to IGF signaling (27–29). It is conceivable that the role of these proteins or others could be more important than BCRP as a resistance mechanism to benzimidazole or IGF targeting agents in general.

Clinical development of novel agents targeting the IGF pathway is ongoing in a variety of tumor types (7). Among the first agents investigated are monoclonal antibodies against IGF-1R, which were not explored as part of our work described above and likely have different mechanisms of resistance (30–32). Although it is unlikely that BCRP will have a role in the determination of sensitivity to anti-IGF-1R monoclonal antibodies, it is less clear if non-benzimidazole IGF-1R tyrosine kinase inhibitors will be substrates for efflux. BMS-754807 is a non-benzimidazole small molecule inhibitor of IGF-1R and InsR with low nanomolar potency (21, 22). BMS-754807 does not appear to induce upregulation of BCRP. These data suggest that BMS-754807 has different potential mechanisms of resistance compared with IGF-1R tyrosine kinase inhibitors derived from a benzimidazole structure. Currently, BMS-754807 is in clinical development in early-phase studies.

**Disclosure of Potential Conflicts of Interest**

F. Huang, J.M. Carboni, and M.M. Gottardis are employees of Bristol Meyers Squibb; P. Haluska is an unpaid consultant for Bristol Meyers Squibb; and D.D. Ross has patent interests in BCRP.

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