

# Differential Mechanisms of Acquired Resistance to Insulin-like Growth Factor-I Receptor Antibody Therapy or to a Small-Molecule Inhibitor, BMS-754807, in a Human Rhabdomyosarcoma Model

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## Abstract

Agents targeting insulin-like growth factor-I receptor (IGF-IR), including antibodies and small-molecule inhibitors, are currently in clinical development for the treatment of cancers including sarcoma. However, development of resistance is a common phenomenon resulting in failures of anticancer therapies. In light of this problem, we developed two resistant models from the rhabdomyosarcoma cell line Rh41: Rh41-807R, with acquired resistance to BMS-754807, a small-molecule dual-kinase inhibitor targeting IGF-IR and insulin receptor (IR), and Rh41-MAB391R, with resistance to MAB391, an IGF-IR–blocking antibody. In addition, tumor xenograft models were established from Rh41 and Rh41-807R cell lines. Gene expression and DNA copy number analyses of these models revealed shared as well as unique acquired resistance mechanisms for the two types of IGF-IR inhibitors. Each resistant model used different signaling pathways as a mechanism for proliferation. Platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) was amplified, overexpressed, and constitutively activated in Rh41-807R cells and tumors. Knockdown of PDGFR $\alpha$  by small interfering RNA in Rh41-807R resensitized the cells to BMS-754807. Synergistic activities were observed when BMS-754807 was combined with PDGFR $\alpha$  inhibitors in the Rh41-807R model *in vitro*. In contrast, AXL expression was highly elevated in Rh41-MAB391R but downregulated in Rh41-807R. Notably, BMS-754807 was active in Rh41-MAB391R cells and able to overcome resistance to MAB391, but MAB391 was not active in Rh41-807R cells, suggesting potentially broader clinical activity of BMS-754807. This is the first study to define and compare acquired resistance mechanisms for IGF-IR–targeted therapies. It provides insights into the differential acquired resistance mechanisms for IGF-IR/IR small-molecule inhibitor versus anti-IGF-IR antibody. *Cancer Res*; 70(18); 7221–31. ©2010 AACR.

## Introduction

Activation of insulin-like growth factor-I receptor (IGF-IR) signaling contributes to proliferation, survival, angiogenesis, metastasis, and resistance to anticancer therapies in many human malignancies (1), supporting the IGF system as an attractive therapeutic target. The IGF system consists of closely related receptors, the type I and type II IGF receptors (IGF-IR and IGF-IIIR), two insulin receptor (IR) isoforms (IR-A and IR-B), three ligands (IGF-I, IGF-II, and insulin), and several IGF-binding proteins (IGFBP1–IGFBP6). IGF-IR/IR

hybrid receptors have recently been implicated in cancer and are activated by IGF-I and IGF-II with signals similar to the IGF-IR homoreceptors (2–4). IR plays an important role in regulating IGF action and contributes to resistance against IGF-IR–targeted therapies (5). Inhibition of both IGF-IR and IR may be necessary to completely disrupt the malignant phenotype regulated by this signaling pathway (6). The IGF system is becoming one of the most intensively investigated molecular targets in oncology. Currently, there are close to 30 drug candidates targeting the IGF-IR or IGF-IR/IR by antibodies or tyrosine kinase inhibitors (TKI), and a number of them are in clinical trials (7–9).

BMS-754807 is a potent and reversible small-molecule TKI of the IGF-IR/IR family kinases having a wide spectrum of antitumor efficacy (10). Targeting IGF-IR/IR signaling with BMS-754807 has resulted in cancer cell growth inhibition both *in vitro* and *in vivo*. This drug is currently in phase I development for the treatment of a variety of human cancers; preclinically defined efficacious exposures have been achieved with p.o. administration of single, tolerable doses in humans (11), and effects of BMS-754807 on pharmacodynamic biomarkers have been observed in cancer patients (12). Ongoing clinical studies with BMS-754807 will soon provide data on its antitumor activity in cancer patients.

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-0391

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As seen for other cancer drugs, tumors may have the potential to develop resistance to both IGF-IR antibodies and small-molecule inhibitors. There are two fundamental forms of drug resistance: *de novo* resistance, which refers to the failure to respond to the initial treatment of a drug, and acquired resistance, which refers to the relapse of drug treatment after an initial response. The mechanisms of acquired drug resistance are studied by two strategies: one is the molecular analysis of clinical specimens from patients who initially had benefit to treatment but then relapsed on the drug, and another is through *in vitro* cell culture modeling. The latter involves culturing drug-sensitive tumor-derived cell lines in increasing concentrations of the drug until most cells are eliminated and the cultures are eventually enriched with drug-resistant cell populations. The resistant cell lines can then be characterized by genomic approaches to identify resistance mechanisms (13, 14).

To assess the mechanisms of acquired resistance to IGF-IR-targeted therapies, such as TKIs and IGF-IR-blocking antibodies, we used the rhabdomyosarcoma cell line Rh41, which only expresses IGF-IR but no detectable IR, to develop two cell lines. Rh41-807R is resistant to BMS-754807, and Rh41-MAB391R is resistant to MAB391, an IGF-IR neutralizing antibody that competes with IGF-I binding to IGF-IR and can efficiently reduce IGF-IR level by receptor internalization/degradation, reduce phospho (p)-IGF-IR and pAKT in tumor cells (15), and inhibit proliferation of Rh41 (10). Gene expression profiling and DNA copy number analysis were performed on the Rh41, Rh41-807R, and Rh41-MAB391R cell lines to identify the molecular basis underlying the shared mechanisms of acquired resistance to both BMS-754807 and MAB391 as well as unique resistance mechanisms to either drug. This report is the first to define and compare resistance mechanisms for the IGF-IR/IR TKI BMS-754807 and the anti-IGF-IR antibody MAB391. The results provide insights into the differentiation of IGF-IR-targeted therapies and potential strategies toward the reversal or prevention of resistance.

## Materials and Methods

### Establishment of resistant cell lines to IGF-IR inhibitors

The human rhabdomyosarcoma cell line Rh41 was provided by Dr. Lee Helman (National Cancer Institute, Frederick, MD), grown in the medium described previously (16) and tested by SNP 6.0 array analysis for authentication. To develop cells with acquired resistance to either BMS-754807 or MAB391 (R&D Systems, Inc.), Rh41 cells were initially exposed to each drug at their corresponding  $IC_{50}$  concentration and then at gradually increasing concentrations of every other culture passage. The  $IC_{50}$  values of the cells to the drugs were determined periodically during this treatment time until the resistance level reached a plateau.

### *In vitro* cellular proliferation assays

Cell proliferation was evaluated by [ $^3H$ ]thymidine incorporation after exposure to either BMS-754807 or MAB391 for 72 hours as described previously (10).

### Gene expression profiling

RNA was isolated from cells using RNeasy kits from Qiagen to generate gene expression data using Affymetrix HT-HG-U133A GeneChip (Affymetrix) according to the manufacturer's instructions. Microarray data were analyzed by ANOVA using Partek to identify genes differentially expressed between resistant and sensitive cell lines.

### Quantitative reverse transcription-PCR (qRT-PCR)

The TaqMan gene expression assay reagents for *IGF-IR*, platelet-derived growth factor receptor  $\alpha$  (*PDGFR $\alpha$* ), fibroblast growth factor receptor 2 (*FGFR2*), and  $\beta$ -actin genes were purchased from Applied Biosystems. The assays were performed according to the manufacturer's protocol. The mRNA expression level was normalized to  $\beta$ -actin expression.

### Gene copy analysis

DNA was isolated from  $5 \times 10^6$  cells using the DNeasy blood and tissue kit from Qiagen. Two aliquots of 250 ng of genomic DNA per sample were digested by *NspI* and *StyI* restriction enzymes, respectively. The resulting products were ligated to the corresponding adaptors and PCR amplified. The labeled PCR products were hybridized to the Human SNP 6.0 array according to the Affymetrix recommendations. The Cel files were processed using an *aroma.affymetrix* package (17) in the R project. Segmentation of normalized raw copy number data was performed with the CBS algorithm (18) implemented in the *aroma.affymetrix* package. Copy number gain (or loss) of a gene was obtained by using the maximum (or minimum) of segmented copy number values within the genomic region of the gene.

### Western blot analyses

Antibodies against pIGF-IR/pIR, pAKT, phospho-extracellular signal-regulated kinase (pERK), and PDGFR $\alpha$  were purchased from Cell Signaling Technology. Total IGF-IR and  $\beta$ -actin antibodies were obtained from Santa Cruz and Chemicon International, respectively. Cell or tumor lysates were analyzed as previously described (10). The signal intensity was scanned and quantified.

### Flow cytometry for IGF-IR expression

Rh41-MAB391R cells were seeded in six-well plates in duplicate with or without 3 nmol/L MAB391 and cultured overnight. The medium was removed, and fresh medium without the MAB391 was added. Cells were collected at 0.5, 1, 2, 4, 16, and 24 hours after washout, stained with IGF-IR antibody (Becton Dickinson), and analyzed by FACScan (FACSCalibur, Becton Dickinson).

### Small interfering RNA

Rh41 and Rh41-807R cell transfections were carried out using ON-TARGETplus small interfering RNA (siRNA) to human PDGFR $\alpha$  (Dharmacon) with DharmaFECT transfection reagents and Opti-MEM medium (Invitrogen) according to the DharmaFECT General Transfection Protocol. RISC-free

siRNA (Dharmacon) was transfected into cells as the negative control. After the addition of the drug, cells were incubated at 37°C for 72 hours, then cell proliferation was measured by [<sup>3</sup>H]thymidine incorporation.

### ***In vivo* xenografts**

Mice were given a subcutaneous implant of a tumor fragment (~20 mg) with a 13-gauge trocar. Tumors were allowed to grow to the predetermined size window (150–175 mg). The mice were then randomized to receive either vehicle or BMS-754807 at 50 mg/kg for 14 days (eight mice per group). Tumor volume and body weight were measured to assess tumor growth inhibition as described previously (10).

## **Results**

### **Rh41-807R is cross-resistant to other IGF-IR inhibitors including MAB391, but Rh41-MAB391R is still sensitive to BMS-754807**

The Rh41 cell line was chosen for induction of acquired resistance because it expresses IGF-IR (16), the target of both BMS-754807 and MAB391, and is sensitive to both drugs (10). Using stepwise exposure to increasing concentrations of either BMS-754807 (~5 months) or MAB391 (~3 months), the cell lines with acquired resistance to either agent were developed and designated as Rh41-807R and Rh41-MAB391R, respectively. Because the highest BMS-754807 concentration used to develop Rh41-807R was 600 nmol/L, which is 120-fold higher than the IC<sub>50</sub> of BMS-754807 for Rh41, the drug at this dose level could potentially have activities against other kinases, such as Met, TrkA, and TrkB (10). Isolation of resistant, single-cell clones was not feasible because Rh41 cells grow poorly at low densities; therefore, resistant cell lines (Rh41-807R or Rh41-MAB391R) represented pooled populations. No significant differences were seen between *in vitro* growth rates in these cell lines (Fig. 1A).

Cell proliferation studies were conducted to compare the sensitivity of these cell lines. The parental Rh41 cells were sensitive to both BMS-754807 (IC<sub>50</sub> = 5 nmol/L) and MAB391 (IC<sub>50</sub> = 0.1 nmol/L); Rh41-807R cells had nearly a 162-fold increase in IC<sub>50</sub> in response to BMS-754807, and Rh41-MAB391R cells had a >10,000-fold increase in IC<sub>50</sub> in response to MAB391 (Table 1). Furthermore, when Rh41-807R cells were out of drug selection for a period of 3 months (Rh41-807Rout), they still displayed resistance to BMS-754807 with an IC<sub>50</sub> of 465 nmol/L, suggesting that the resistance of Rh41-807R to BMS-754807 was persistent.

Intriguingly, BMS-754807 was active against Rh41-MAB391R cells, whereas MAB391 was unable to inhibit the growth of either Rh41-MAB391R or Rh41-807R even at a concentration of >2 μmol/L (Table 1). In addition, Rh41-807R showed cross-resistance to other multiple IGF-IR/IR TKIs; however, Rh41-MAB391R displayed sensitivity to these drugs (Table 1). These results suggest that there may be distinct mechanisms of resistance to BMS-754807 and MAB391.

When Rh41-807R was tested against inhibitors of epidermal growth factor receptor (EGFR) family kinases or MET kinase, it retained similar response levels as the parental

Rh41 cells to these targeted agents (data not shown). In addition, when Rh41-807R was tested against multiple cytotoxic agents as well as the mammalian target of rapamycin inhibitor, the cells maintained a very similar level of sensitivity as the Rh41 cell line (Supplementary Table S1), suggesting that Rh41-807R was specifically resistant to IGF-IR inhibitors and the mechanism of resistance to IGF-IR inhibitors did not affect the responsiveness to cytotoxic agents.

### **IGF-IR expression and activity contributes to acquired resistance to IGF-IR inhibition**

Because both BMS-754807 and MAB391 target IGF-IR, we investigated whether changes in IGF-IR expression and pathway activity are involved in the development of acquired resistance. Compared with the sensitive Rh41 cells, similar IGF-IR RNA and protein expression levels were maintained in Rh41-MAB391R cells, but downregulated levels were found in Rh41-807R cells (Fig. 1B). IR is not expressed in all cell lines and may not contribute to the resistance to either BMS-754807 or MAB391. Although RNA expression levels of IGF-IIR and IGF-II were downregulated in Rh41-807R cells (Supplementary Fig. S1), IGF-IIR protein levels seem to be similar in all cell lines (Fig. 1B). Interestingly, insulin receptor substrate-1 (IRS-1) protein was also downregulated in Rh41-807R (Fig. 1B).

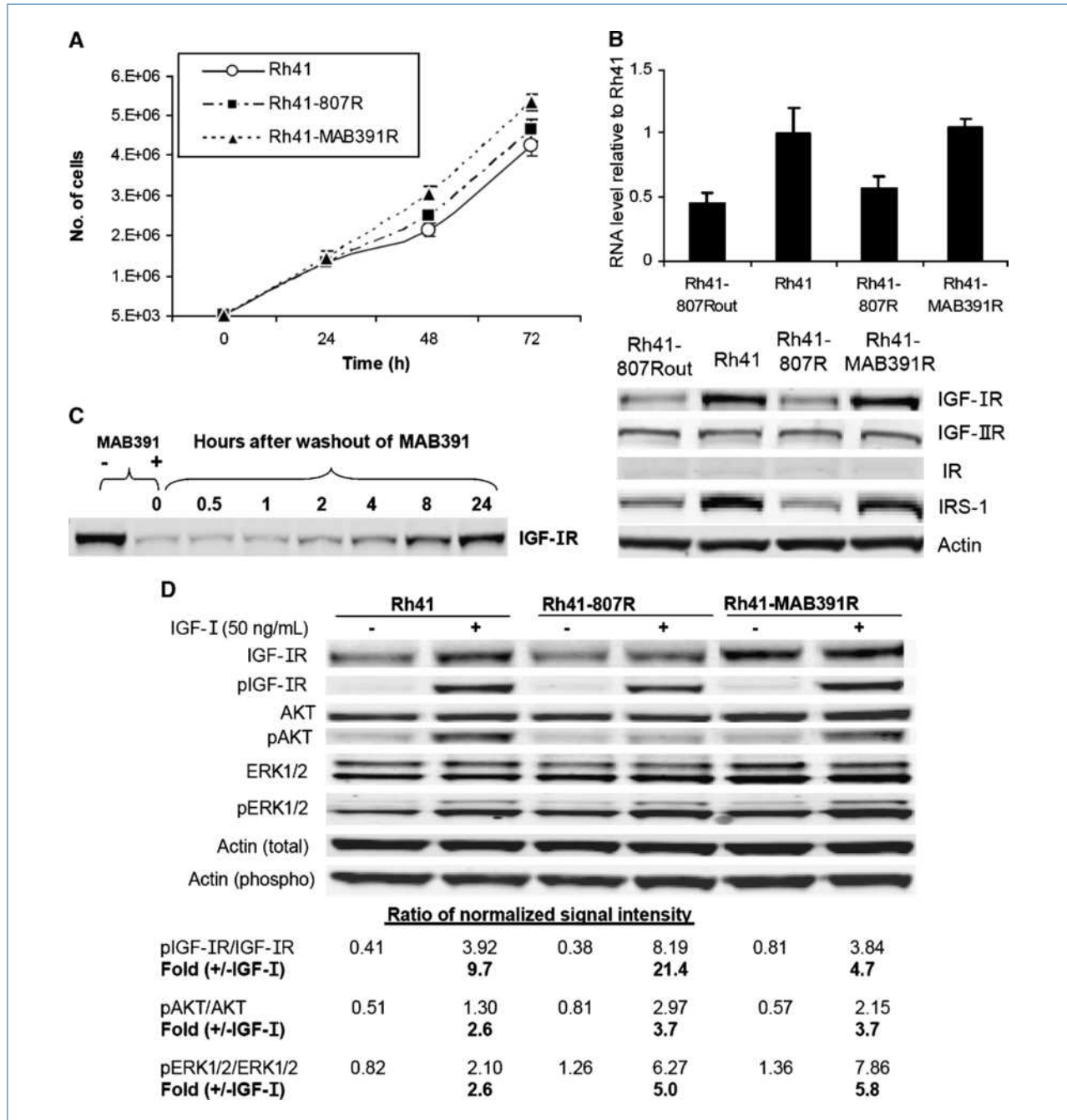
In Rh41-MAB391R cells, IGF-IR protein decreased in the presence of MAB391; both total (Fig. 1C) and cell surface protein levels (Supplementary Fig. S2) began to recover 2 hours after removal of MAB391 and returned to normal level by 24 hours. However, for Rh41-807R cells, both IGF-IR RNA and protein did not return to control levels after removal of BMS-754807 for even 3 months (Rh41-807Rout in Fig. 1B). BMS-754807 may cause transcriptional regulation of IGF-IR, whereas MAB391 induces IGF-IR internalization/degradation (15), leading to a subsequent decrease in IGF-IR cell surface expression in Rh41-MAB391R cells. The rapid restoration of IGF-IR protein level after MAB391 removal may then be a consequence of the unchanged levels of RNA, which is continuously available for translation.

We also investigated whether there is any difference in IGF-IR phosphorylation and activation of downstream signaling pathways such as pAKT and pERK in response to IGF-I stimulation between Rh41, Rh41-MAB391R, and Rh41-807R cell lines (Fig. 1D). In the absence of IGF-I, all cell lines showed low to undetectable pIGF-IR activity, but all responded to IGF-I stimulation by showing an increase in pIGF-IR, pAKT, and pERK. Quantitative analysis (Supplementary Fig. S3; Fig. 1D) showed that Rh41-807R had much higher fold increases in the IGF-I–stimulated versus non-stimulated pIGF-IR/IGF-IR ratio (21.4-fold) than Rh41 (9.7-fold), whereas Rh41-MAB391R only had 4.7-fold, which was lower than that of Rh41. Both Rh41-807R and Rh41-MAB391R cells had higher basal levels of pERK/ERK ratio than Rh41 cells, indicating higher basal activation of signaling in resistant models. Both resistant cell lines had similar increases in pERK and pAKT than the parental cells in response to IGF-I stimulation, suggesting no significant difference in AKT and ERK activation through IGF signaling in both resistant cell lines.

### Shared and unique gene expression alterations between Rh41-807R and Rh41-MAB391R

To explore the molecular differences between the sensitive and acquired resistant models, gene expression profil-

ing was performed using Affymetrix GeneChips. Statistical analyses of gene expression profiles identified two sets of gene lists: one list included genes that are differentially expressed in Rh41-807R versus parental Rh41 (upregulated



**Figure 1.** *in vitro* growth characterization of cell lines. Cells were seeded at 5,000 per well; trypsinized at 24, 48, and 72 h postseeding; and counted from triplicate wells. Points, average cell numbers three independent experiments; bars, SD. B, differential expression patterns of IGF-IR RNA and IGF pathway proteins as measured by qRT-PCR or Western blot analyses. C, recovery of IGF-IR protein expression in Rh41-MAB391R cells after washout of MAB391. D, comparison of signaling in response to IGF-I stimulation between Rh41-807R, Rh41-MAB391R, and Rh41 cells. The signal intensity of each blot was assessed quantitatively and normalized to  $\beta$ -actin. Activation of IGF-IR, AKT, and ERK1/2 was expressed as the normalized ratio of phosphorylated protein/total protein. The fold changes between IGF-I-stimulated versus nonstimulated conditions with pIGF-IR/IGF-IR, pAKT/AKT, and pERK/ERK ratios were calculated.



**Table 1.** Resistance characterization of the Rh41-807R and Rh41-MAB391R models to multiple IGF-IR inhibitors

Inhibitors	IC <sub>50</sub> (nmol/L)		
	Rh41	Rh41-807R	Rh41-MAB391R
BMS-754807	5	811	27
MAB391	0.1	>2,000	>2,000
BMS-536924	31	>1,000	90
BMS-830913	0.02	9	0.02
BMS-809800	2	55	1.29
BMS-808931	<1	28	<0.64

NOTE: The sensitivity/resistance is defined as IC<sub>50</sub>, which is the drug concentration that produced a 50% growth inhibition compared with untreated controls in [<sup>3</sup>H]thymidine incorporation assays.

and downregulated) and the other list included genes differentially expressed in Rh41-MAB391R versus parental Rh41. Comparison of these two gene lists (Fig. 2A) identified three groups of genes.

The first group of genes with a similar expression pattern in both Rh41-807R and Rh41-MAB391R (sections A and D of Fig. 2A and B; Supplementary Table S2) may contribute to shared mechanisms of acquired resistance to agents targeting IGF-IR. These genes are involved in cell signaling pathways (e.g., *FGF9*, *PDGFRα*, and *DUSP13*), cell matrix interactions (e.g., *MMP2*, *MMP3*, and *TIMP3*), cell cycle regulation (e.g., *DCX*, *PLAU*, *AMACR*, and *MXI1*), and apoptosis (e.g., *PAWR*, *PAX3*, and *TMSB4X*). Compared with sensitive Rh41 cells, *MMP2*, *TIMP3*, and *FGF9* were upregulated, whereas *SNRPN*, *TFAP2B*, *MMP3*, and *PLAU* were downregulated, in both Rh41-807R and Rh41-MAB391R cells.

The second group of genes had opposite expression changes between Rh41-807R and Rh41-MAB391R cells (sections B and C of Fig. 2A and B; Supplementary Table S3). Genes such as *AXL*, *FADS3*, *MME*, *NNMT*, and *PLXNC1* were upregulated in Rh41-MAB391R but downregulated in Rh41-807R, whereas *MYO22*, *EPHA3*, and *CDH2* genes had converse patterns.

The third group of genes showed expression changes only in one resistant model (genes in sections E and F or G and H of Fig. 2A). For example, *DLK1* was upregulated whereas *TUSC3* and *VCAN* were downregulated only in Rh41-MAB391R, but there were no changes observed in Rh41-807R (Supplementary Fig. S4A). *FHL1*, *EEF1A2*, *PRRX*, and *GHR* were upregulated whereas *INSIG1*, *CCND1*, *CCND2*, and *ERBB3* were downregulated in Rh41-807R only (Supplementary Fig. S4B and C).

Genes in the latter two groups may contribute to distinct resistance mechanisms between BMS-754807 and MAB391. Ingenuity pathway analysis was performed for the genes that were uniquely overexpressed in either Rh41-807R (sec-

tions C and E of Fig. 2A) or Rh41-MAB391R cells (sections B and G of Fig. 2A). Biological functions and canonical signaling pathways of the two gene lists involved were then cross-compared. The top 10 are shown in Supplementary Fig. S5. Although these two gene lists are made up of different genes, their contributions to the same molecular and cellular functions were very similar, whereas the canonical pathways these genes were involved in were significantly different. For example, the genes overexpressed in Rh41-807R had higher contribution to the IR and IGF-I, PTEN, PDGF, or EGF signaling pathways, suggesting that these canonical pathways may contribute to differential resistance to BMS-754807.

Overall, expression patterns were similar in both Rh41-807R and Rh41-807Rout (Fig. 2B). Only a limited number of genes in Rh41-807Rout had their expression restored to the levels similar to parental Rh41 cells, suggesting that gene expression patterns in Rh41-807R persisted even after the drugs were removed.

### DNA copy number changes are associated with acquired resistance to BMS-754807 or MAB391

Whole-genome DNA copy number analysis was performed using SNP chip for genomic abnormalities. Many low-level copy number changes (gain or loss) were observed in resistant Rh41-807R cells compared with sensitive Rh41 cells, whereas only a few copy number gains were observed in Rh41-MAB391R cells (Supplementary Fig. S6A; Supplementary Table S4). For example, both Rh41 and Rh41-MAB391R had mostly normal copy numbers on chromosome 4 in the q11-q21 region; however, Rh41-807R had genomic abnormalities in this region (Supplementary Fig. S6B). Rh41-807R harbored amplification of chr4q11-q12, which contains the *c-KIT* and *PDGFRα* genes, with 1.9- and 3.3-fold gains compared with Rh41, respectively (Fig. 2C). Gene amplification was consistent with the overexpression of these two genes seen in Rh41-807R cells (Fig. 3A; Supplementary Fig. S7). Another interesting observation is that PTEN loss was found in Rh41-807R compared with Rh41 cells (Supplementary Table S4), which is consistent with the decreased gene expression level in Rh41-807R cells (1.8-fold reduction,  $P = 0.018$ ; expression data not shown).

### Rh41-807R and Rh41-MAB391R overexpress unique genes in alternative signaling pathways mediating differential resistance mechanisms

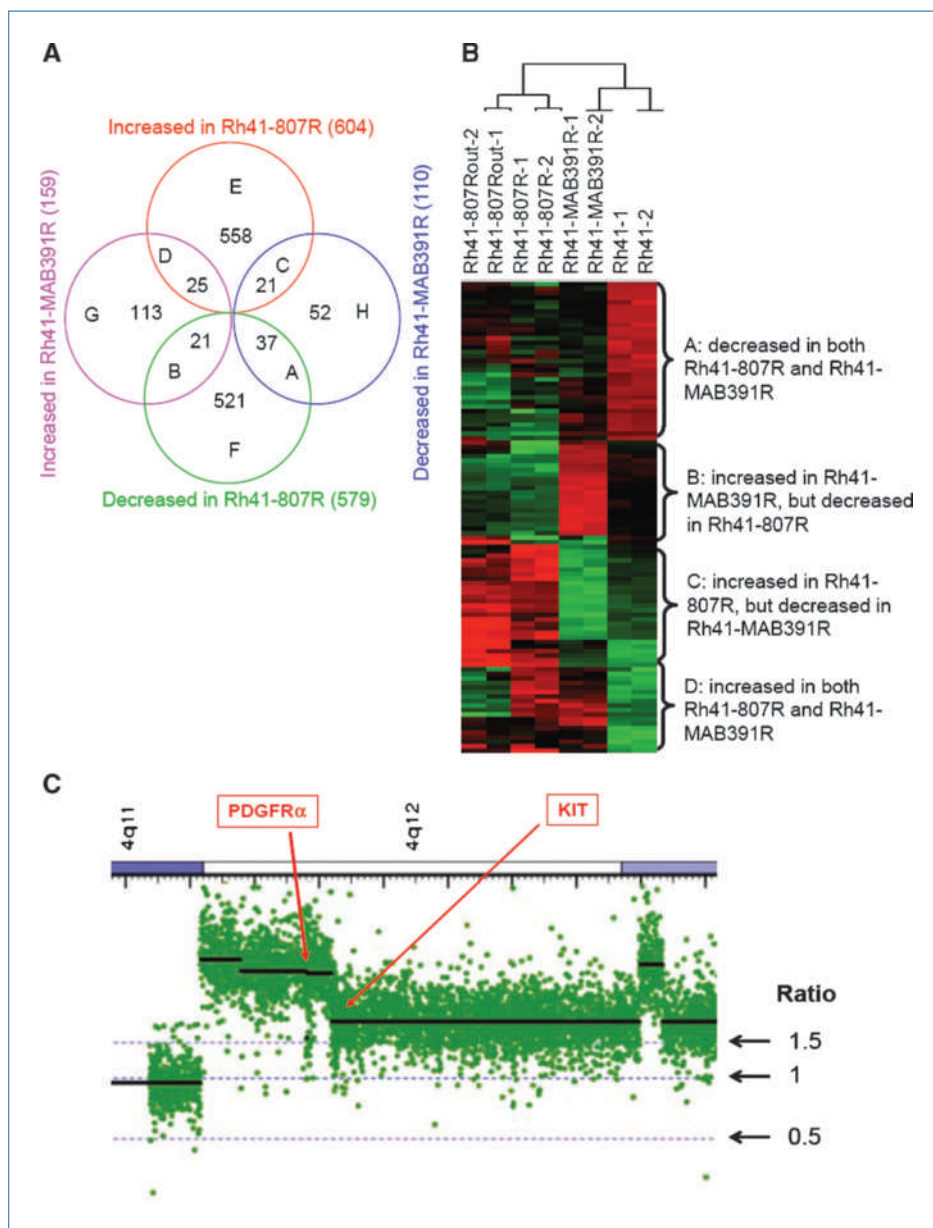
Gene expression profiling identified genes in key signaling pathways that were differentially expressed between Rh41-807R and Rh41-MAB391R cells. PDGFRα showed significant increases of RNA and protein expression in Rh41-807R cells compared with Rh41 cells; this was not seen in Rh41-MAB391R, and the induction of PDGFRα was observed even in cells out of BMS-754807 selection for 3 months (Fig. 3A). PDGFRα was constitutively activated in the presence or absence of the ligand PDGFBB in Rh41-807R cells, suggesting that the activation was ligand independent (Fig. 3B). Although in sensitive Rh41 cells it was undetectable (Fig. 3A), PDGFRα RNA level was induced after treatment with BMS-754807 in a

concentration-dependent manner (Fig. 3C), whereas IGF-IR levels were unchanged (Supplementary Fig. S8). PDGFR $\alpha$  induction was an early event and observable at 4 hours after treatment (Fig. 3C), and the induction was also observed by treatment with two other IGF-IR small-molecule inhibitors (data not show). Other genes, such as *c-KIT*, *FGFR2*, and *EPHA3*, had a similar RNA expression pattern as PDGFR $\alpha$ , whereas c-KIT protein level was similar in these cell lines (Supplementary Fig. S7). Several Eph receptor family members such as *EPHA3*, *EPHA4*, and *EPHB2*, as well as ligand *EFNA3*, were overexpressed in Rh41-807R. A large number of RAS/RAF-related genes (e.g., *ARAF*, *ARHGAP8*, *RAB40B*, *RAB6B*, *RAB7L1*, *RASL11B*, *RASSF2*, *RHOC*, and *RHOQ*) were also upregulated only in Rh41-807R cells (data not shown).

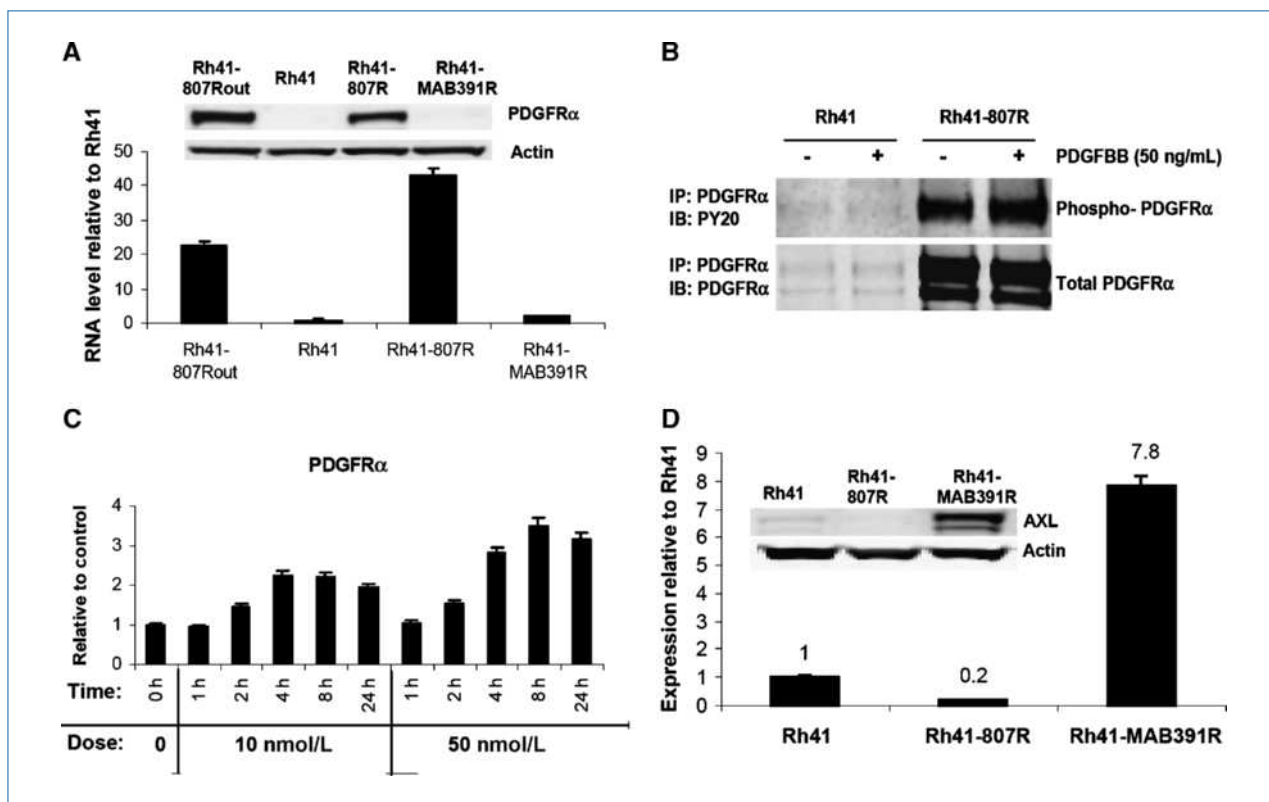
AXL was an interesting example with an opposite expression pattern in resistant models. Its RNA expression level was 7.8-fold higher in Rh41-MAB391R cells but 4.9-fold lower in Rh41-807R cells compared with the sensitive parental Rh41, and Western blot analysis further confirmed that AXL protein expression was increased in Rh41-MAB391R (Fig. 3D).

#### Rh41-807R retains resistance to BMS-754807 *in vivo*

To test whether Rh41-807R is also resistant to BMS-754807 *in vivo*, both Rh41 and Rh41-807R xenograft tumors were established. Rh41 achieved 73% tumor growth inhibition (TGI) in response to BMS-754807, whereas Rh41-807R had only 35% TGI, indicating that the resistance was consistent with the *in vitro* observations (Fig. 4A).



**Figure 2.** A, schematic illustration of a cross-comparison of genes that are commonly or differentially expressed between the Rh41-807R and Rh41-MAB391R cell lines. B, gene expression pattern (two replicates per cell line) for the overlapped genes identified in A. C, amplification of chromosome 4q11-q12 containing the *PDGFR $\alpha$*  and *c-KIT* genes is detected in Rh41-807R cells compared with Rh41 cells.



**Figure 3.** A, PDGFR $\alpha$  RNA and protein expression is upregulated in Rh41-807R but not in Rh41-MAB391R, as measured by quantitative RT-PCR and Western blot, respectively. B, overexpression and constitutive activation of PDGFR $\alpha$  protein in Rh41-807R cells. Cells were starved for 24 h (0.3% bovine serum albumin) and then stimulated with or without the ligand PDGFBB (PeproTech) for 5 min. Total protein (500  $\mu$ g) was immunoprecipitated (IP) with PDGFR $\alpha$  antibody and analyzed on SDS gel. Membrane was immunoblotted (IB) with phosphotyrosine PY20 (BD Biosciences) and total PDGFR $\alpha$ . C, PDGFR $\alpha$  transcript induction by BMS-754807 in Rh41 cells is an early event and dose dependent. PDGFR $\alpha$  RNA levels were measured by quantitative RT-PCR and compared with the untreated time 0 control. D, AXL RNA and protein expression is upregulated in Rh41-MAB391R but downregulated in Rh41-807R cells.

Examination of the expression of IGF-IR and PDGFR $\alpha$  in these tumors revealed that the reduction in IGF-IR and the increase in PDGFR $\alpha$  protein expression in Rh41-807R tumors were retained (Fig. 4B). Rh41-807R tumors also had higher RNA expression levels of c-KIT, FGFR2, and PDGFR $\alpha$  compared with Rh41 tumors, which is consistent with the pattern observed in cell lines (Fig. 4C), suggesting that the mechanisms of resistance to BMS-754807 were maintained *in vitro* and *in vivo*. It was noticed that the fold increase of PDGFR $\alpha$  in Rh41-807R tumors was relatively lower than that in the cell line. This could be a result of the mice not being treated with BMS-754807 during the time between tumor implantation and reaching the predetermined size, and thus it actually mimicked the Rh41-807Rout situation.

#### Modulation of PDGFR $\alpha$ activity or expression alters the response to BMS-754807 in Rh41-807R cells

Because PDGFR $\alpha$  expression was upregulated and constitutively activated in Rh41-807R cells, we tested whether dovitinib, a drug that has activity against PDGFR, is able to inhibit the growth of the resistant cells. Rh41-807R cells were more sensitive to dovitinib than Rh41 (Fig. 5A), suggesting

that PDGFR may play a role in the resistance to BMS-754807. Considering that dovitinib inhibits other kinases such as vascular endothelial growth factor receptor, c-KIT, FGFR, and FLT (19) and Rh41-807R cells also had increased RNA expression of c-KIT and FGFR2 (Supplementary Fig. S7) in addition to PDGFR $\alpha$ , the ability of dovitinib to target multiple kinases may contribute to the increased sensitivity of Rh41-807R to the compound. We tested several compounds (axitinib, sorafenib, and sunitinib) that have activity against PDGFR and observed varying degrees of growth inhibitory activity in Rh41-807R cells (Supplementary Table S5), again suggesting that PDGFR is involved in mediating resistance to BMS-754807.

To further examine the correlation between the overexpression of PDGFR $\alpha$  and the acquisition of BMS-754807 resistance, we suppressed the expression of PDGFR $\alpha$  using siRNA-mediated knockdown of PDGFR $\alpha$  in Rh41-807R cells. The PDGFR $\alpha$ -specific siRNA was able to efficiently suppress the expression of PDGFR $\alpha$  (Fig. 5B), whereas it had no effect on IGF-IR expression (Supplementary Fig. S9). We next studied the effect of PDGFR $\alpha$  suppression on response to BMS-754807 using an *in vitro* cell proliferation assay. Both Rh41

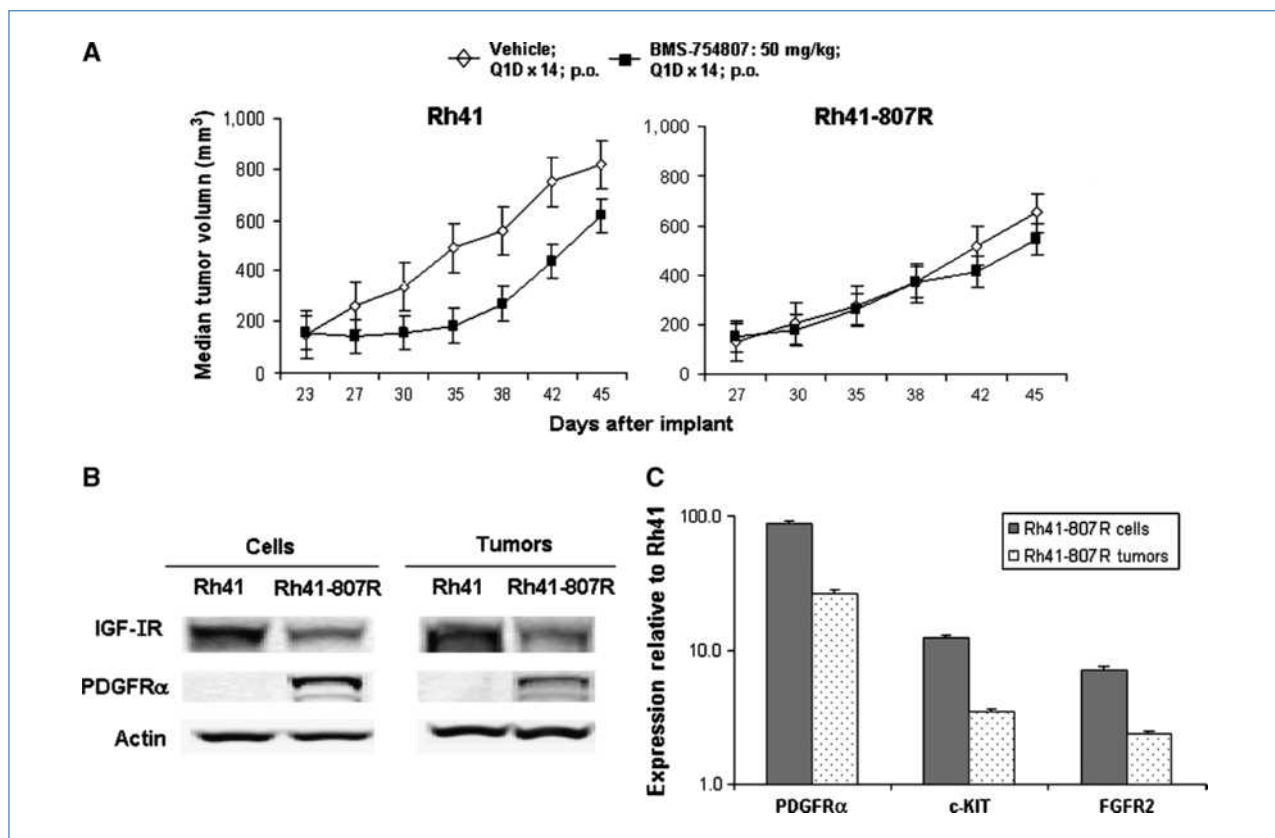
and Rh41-807R cells were transfected with either control or PDGFR $\alpha$ -specific siRNA, followed by treatment with different concentrations of BMS-754807 for 72 hours. Rh41 cells were still sensitive to BMS-754807, with no differences observed in the sensitivity between untransfected, control siRNA-transfected, and PDGFR $\alpha$  siRNA-transfected cells (Fig. 5B), probably due to the very low level of PDGFR $\alpha$  expression (Fig. 3A). Rh41-807R cells transfected with PDGFR $\alpha$  siRNA restored growth inhibition sensitivity to BMS-754807 to similar levels as Rh41 cells, whereas control siRNA-transfected cells were still resistant to BMS-754807 with no change in sensitivity (Fig. 5B). These results provided evidence that overexpression of PDGFR $\alpha$  confers resistance to BMS-754807 and that downregulation of PDGFR $\alpha$  in Rh41-807R cells restores sensitivity to BMS-754807.

We next tested the effect of combining BMS-754807 with PDGFR antagonists in Rh41-807R cells. BMS-754807 alone was not active, and dovitinib, sunitinib, imatinib, or axitinib had varying degrees of activity in Rh41-807R cells; combinations of BMS-754807 with any of these PDGFR inhibitors had synergistic effects on growth inhibition (Supplementary Fig. S10). This indicated that inhibition of both IGF-IR and PDGFR may be necessary to inhibit the proliferation of cells that had acquired resistance to IGF-IR inhibitors mediated by PDGFR activation.

## Discussion

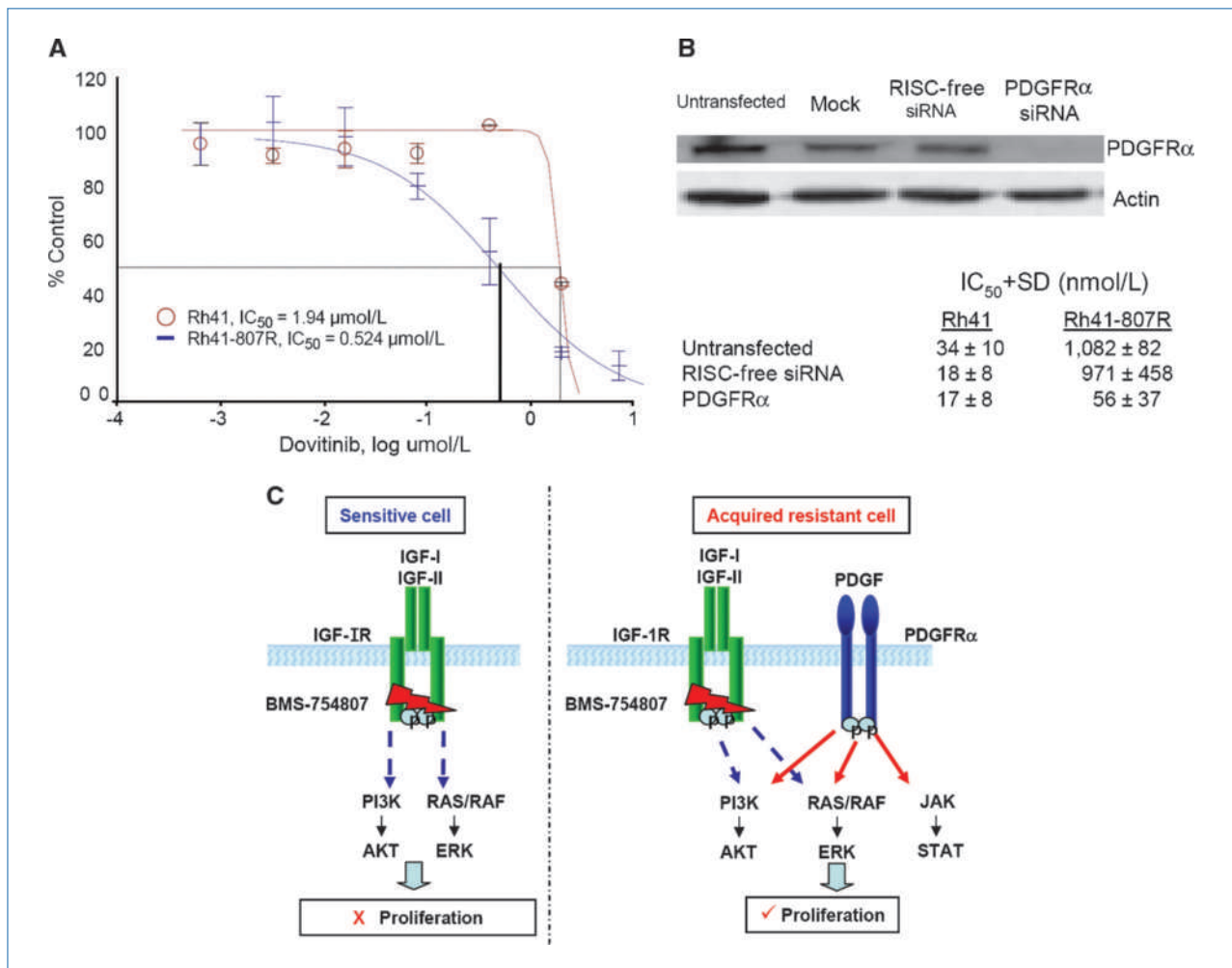
Multiple resistance mechanisms compromise the successful clinical application of inhibitors targeting oncogenic tyrosine kinases (13, 14). As both antibodies and TKIs targeting IGF-IR are currently in clinical testing, it is important to understand the mechanisms of resistance to IGF-IR inhibitors so that rational therapy strategies can be defined to reverse or prevent resistance. In addition, biomarkers of resistance will help to identify those tumors more likely to respond to other specific agents. The aim of this study was to identify the mechanisms of acquired resistance to IGF-IR-targeted therapies. To achieve this, cell lines with acquired resistance to either BMS-754807 or the IGF-IR antibody MAB391 were developed using Rh41 rhabdomyosarcoma cancer cells *in vitro* to compare and define the commonalities and differences in resistance mechanisms between these agents.

Accumulating evidence suggests that similar molecular mechanisms could underlie both *de novo* resistance and acquired resistance (13, 14). As we previously reported in a sarcoma cell line panel, the level of IGF-IR expression was associated with response to IGF-IR inhibitors, and lower expression of IGF-IR was seen in more resistant cell lines (16).



**Figure 4.** *In vivo* response to BMS-754807 in Rh41 and Rh41-807R xenografts. Eight tumor-bearing mice per group were treated at the dose and schedule indicated. B, comparison of IGF-IR and PDGFR $\alpha$  protein expression in Rh41 and Rh41-807R cells and tumors. C, RNA expression levels of PDGFR $\alpha$ , c-KIT, and FGFR2 in cell lines and tumors.





**Figure 5.** PDGFR $\alpha$  confers resistance to BMS-754807 in Rh41-807R cells. **A**,  $IC_{50}$  curves for dovitinib as determined by an *in vitro* proliferation assay. **B**, PDGFR $\alpha$  expression knockdown by siRNA regains the sensitivity to BMS-754807 in Rh41-807R cells 72 h after transfection. The average  $IC_{50}$  values and SD from three independent experiments are shown. **C**, schematic illustration of acquired resistance mechanisms to BMS-754807. Left, cells with high expression of IGF-IR and activated pathway show response to BMS-754807, with inhibition of downstream effectors and consequent inhibition of proliferation. Right, cells with acquired resistance to IGF-IR inhibitor arise through amplification of PDGFR $\alpha$  and, subsequently, kinase activation, which transduces the overlapped proliferation signals.

This was further confirmed by a recent report that IGF-IR levels correlated with sensitivity to an anti-IGF-IR–targeting antibody in breast and colon cell lines (20). In the present study, we observed that downregulation of IGF-IR in the cells acquired resistance to BMS-754807 (Fig. 1B) albeit via different mechanisms of action. The mechanism by which BMS-754807 causes the chronic loss of IGF-IR expression is an interesting question, and it could potentially be through transcriptional regulation and protein degradation. Low expression of IGF-IR may possibly lead to fewer membrane receptors, lower IRS-1 levels, and, consequently, less dependence on the IGF pathway for growth; although it still responds to IGF-I stimulation for signaling (Fig. 1D), it is much less sensitive to BMS-754807 for proliferation (Table 1) possibly due to activation of other pathways. This may serve as one of the resistance mechanisms for both *de novo* and

acquired resistance to IGF-IR inhibitors. In addition, there is a possibility that IGF-IR gene mutations evolved during the process of acquiring resistance, which could be monitored in a clinical setting.

Genetic alterations that lead to overexpression or altered function of gene products in tumors have been implicated in drug resistance (13). This includes an acquisition of additional copies of specific genes and/or induction of alternative receptor signaling molecules resulting in a switch to other signaling pathway dependencies in the acquired resistance to TKIs. One example of this switch is the amplification or the induction of MET kinase in non–small cell lung cancer patients or cells with acquired resistance to EGFR TKI therapy (21, 22). We found DNA copy number gains in Rh41-807R cells in chr4q11-q12, where PDGFR $\alpha$  and c-KIT are located (Fig. 2C). Overexpression of PDGFR $\alpha$  seems to precede

amplification of the gene. Induction of PDGFR $\alpha$  RNA in Rh41 cells was an early event and seen at 4 hours after BMS-754807 treatment (Fig. 3C). The overexpression of PDGFR $\alpha$  in Rh41-807R (Fig. 3A) and the ability of PDGFR $\alpha$  siRNA to restore BMS-754807 sensitivity in Rh41-807R cells (Fig. 5B) provide strong evidence that PDGFR $\alpha$  is involved in resistance to BMS-754807 in this model system. PDGFR $\alpha$  amplification in tumors has been previously reported, especially in glioblastomas and sarcoma (23, 24), but its role in drug resistance has not been previously described. This is the first study linking the amplification/overexpression of PDGFR $\alpha$  with resistance to targeted inhibitors.

The enhancement of tumor cell proliferation and the induction of resistance to therapy via cross-talk between the IGF-IR and PDGFR pathways are not fully understood. There are overlaps in a common set of downstream effectors of IGF-IR and PDGFR $\alpha$  signaling. These consist of phosphoinositide 3-kinase/AKT and RAS/RAF/MEK/ERK signaling molecules, which are integrators of growth signals originating from IGF-IR or PDGFR $\alpha$ . We hypothesize (Fig. 5C) that, in the sensitive Rh41 cells, IGF-IR is the predominant driver for proliferation via activation of AKT and ERK, making it sensitive to BMS-754807 inhibition, whereas in Rh41-807R cells, gene overexpression/amplification of the alternate receptor tyrosine kinase PDGFR $\alpha$  leads to its activation, resulting in an increase in proliferation signals through cross-talk between the two pathways. Although targeting IGF-IR still inhibits IGF-IR activation, it does not sufficiently affect the downstream pathways. Inactivation of common proteins that are key transmitters in the IGF-IR and PDGFR $\alpha$  signaling pathways is required to interrupt growth signaling. The combined targeting of these two redundant signaling pathways achieves enhanced inhibition of tumor cell growth (Supplementary Fig. S10). Strategies including combination treatments and biomarker development should be further considered when developing targeted agents to prevent or delay the occurrence of acquired resistance.

We observed an overexpression of AXL in Rh41-MAB391R cells but not in Rh41-807R cells (Fig. 3D). The reason for this difference remains unclear. Recent studies reported that increased expression of AXL may confer acquired resistance to imatinib in gastrointestinal tumors (25), to lapatinib in breast tumor cells (26), and to chemotherapy drugs in acute myeloid leukemia (27).

It is difficult to predict differences in efficacy between anti-receptor antibodies and TKIs due to the disparity in selectivity (28). This has been addressed by comparing erlotinib and cetuximab for targeting EGFR or lapatinib and trastuzumab for targeting HER2 (29). Data from our study indicate that the mechanisms of resistance to BMS-754807 differ from that of the IGF-IR antibody MAB391. Cells that developed resistance to BMS-754807 are cross-resistant to other IGF-IR inhibitors including the anti-IGF-IR antibody MAB391, but cell lines resistant to MAB391 are still sensitive to BMS-754807 (Table 1). This suggests that there could be activity for BMS-754807 in a broader spectrum of tumors, including those that are resistant to IGF-IR antibodies. Patients who fail treatment with IGF-IR antibody therapies may still benefit from BMS-754807 treatment, as it has a wider spectrum against the IGF-IR/IR family and, therefore, may be more effective in inhibiting signaling through both IGF-IR and IR.

In summary, this study offers the first definition and comparison of acquired resistance mechanisms between the TKI BMS-754807 and an antibody against IGF-IR in a human rhabdomyosarcoma Rh41 cells. Acquired resistance to BMS-754807 was associated with increased expression of PDGFR $\alpha$ , whereas resistance to the IGF-IR antibody MAB391 was associated with overexpression of AXL in this model. These molecular changes could serve as biomarkers for the identification of resistant tumors in clinical use. The molecular mechanisms of acquired resistance to IGF-IR inhibitors identified in this study may provide a basis for the rational development of next-generation inhibitors, as well as of effective drug combinations that can overcome or prevent acquired resistance to the inhibitors, thereby enhancing clinical benefit.

## Disclosure of Potential Conflicts of Interest

All authors are employees of Bristol-Myers Squibb Company.

## Acknowledgments

We thank Carmen Raventos-Suarez for technical assistance in flow cytometry analysis and Xi-De Wang for preparing the figures in this article.

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Received 02/14/2010; revised 07/26/2010; accepted 07/27/2010; published OnlineFirst 08/31/2010.

## References

- Samani AA, Yakar S, LeRoith D, et al. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* 2007;28:20–47.
- Morrione A, Valentini B, Xu SQ, et al. Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc Natl Acad Sci U S A* 1997;94:3777–82.
- Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. *Cytokine Growth Factor Rev* 2005;16:421–39.
- Pandini G, Vigneri R, Costantino A, et al. Insulin and insulin-like growth factor-I (IGF) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. *Clin Cancer Res* 1999;5:1935–44.
- Hendrickson AW, Haluska P. Resistance pathways relevant to insulin-like growth factor-1 receptor-targeted therapy. *Curr Opin Investig Drugs* 2009;10:1032–40.
- Law JH, Habibi G, Hu K, et al. Phosphorylated insulin-like growth factor-I/insulin receptor is present in all breast cancer subtypes and is related to poor survival. *Cancer Res* 2008;68:10238–46.

7. Gualberto A, Pollak M. Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions. *Oncogene* 2009;28:3009–21.
8. Rodon J, DeSantos V, Ferry RJ, Kurzrock R. Early drug development of inhibitors of the insulin-like growth factor-I receptor pathway: lessons from the first clinical trials. *Mol Cancer Ther* 2008;7:2575–88.
9. Weroha SJ, Haluska P. IGF-I receptor inhibitors in clinical trials—early lessons. *J Mamm Gland Biol Neoplasia* 2008;13:471–83.
10. Carboni JM, Wittman M, Yang Z, et al. BMS-754807, a small molecule inhibitor of insulin-like growth factor-1R/IR. *Mol Cancer Ther* 2009;8:3341–9.
11. Clemens PL, Park JS, Cohen MB, et al. BMS-754807, an oral dual IGF-IR/IR inhibitor: first-in-human single-dose study of safety, tolerability, pharmacokinetics, and pharmacodynamics in healthy subjects. Proceedings of the AACR-NCI-EORTC International Conference; 2009 November 15–19, Boston, MA. abstract #101.
12. Desai J, Solomon B, Davis ID, et al. BMS-754807, an oral dual IGF-IR/Insulin receptor (IR) inhibitor: early pharmacodynamic (PD) and positron emission tomography (PET) imaging results from a daily-dose study in cancer subjects. Proceedings of the AACR-NCI-EORTC International Conference; 2009 November 15–19, Boston (MA). abstract #109.
13. Jänne PA, Gray N, Settleman J. Factors underlying sensitivity of cancers to kinase inhibitors. *Nat Rev Drug Discov* 2009;8:709–23.
14. Engelman JA, Settleman J. Acquired resistance to tyrosine kinase inhibitors during cancer therapy. *Curr Opin Genet Dev* 2008;18:73–9.
15. Hailey J, Maxwell E, Koukouras K, Bishop WR, Pachter JA, Wang Y. Neutralizing anti-insulin-like growth factor receptor 1 antibodies inhibit receptor function and induce receptor degradation in tumor cells. *Mol Cancer Ther* 2002;1:1349–53.
16. Huang F, Greer A, Hurlburt W, et al. The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. *Cancer Res* 2009;69:161–70.
17. Bengtsson H, Wirapati P, Speed TP. A single-array preprocessing method for estimating full-resolution raw copy numbers from all Affymetrix genotyping arrays including GenomeWideSNP 5 & 6. *Bioinformatics* 2009;25:2149–56.
18. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics* 2004;5:557–72.
19. Homsy J, Daud AI. Spectrum of activity and mechanism of action of VEGF/PDGF inhibitors. *Cancer Control* 2007;14:285–94.
20. Zha J, O'Brien C, Savage H, et al. Molecular predictors of response to a humanized anti-insulin-like growth factor-I receptor monoclonal antibody in breast and colorectal cancer. *Mol Cancer Ther* 2009;8:2110–21.
21. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–43.
22. McDermott U, Pusapati RV, Christensen JG, Gray NS, Settleman J. Acquired resistance of non-small cell lung cancer cells to MET kinase inhibition is mediated by a switch to epidermal growth factor receptor dependency. *Cancer Res* 2010;70:1625–34.
23. Fleming TP, Saxena A, Clark WC, et al. Amplification and/or over-expression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. *Cancer Res* 1992;52:4550–3.
24. Zhao J, Roth J, Bode-Lesniewska B, Pfaltz M, Heitz PU, Komminoth P. Combined comparative genomic hybridization and genomic microarray for detection of gene amplifications in pulmonary artery intimal sarcomas and adrenocortical tumors. *Genes Chromosomes Cancer* 2002;34:48–57.
25. Mahadevan D, Cooke L, Riley C, et al. A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal tumors. *Oncogene* 2007;26:3909–19.
26. Liu L, Greger J, Shi H, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res* 2009;69:6871–8.
27. Hong CC, Lay JD, Huang JS, et al. Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. *Cancer Lett* 2008;268:314–24.
28. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 2006;33:369–85.
29. Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* 2006;6:714–27.

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## Differential Mechanisms of Acquired Resistance to Insulin-like Growth Factor-I Receptor Antibody Therapy or to a Small-Molecule Inhibitor, BMS-754807, in a Human Rhabdomyosarcoma Model

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*Cancer Res* 2010;70:7221-7231. Published OnlineFirst August 31, 2010.

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