Acquired Resistance to FGFR Inhibitor in Diffuse-Type Gastric Cancer through an AKT-Independent PKC-Mediated Phosphorylation of GSK3β

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Abstract

Preclinical models of diffuse-type gastric cancer (DGC) that reliably predict clinical activity of novel compounds are lacking. To overcome the problem of poor tumor cellularity in DGC, we used cells from malignant ascites to establish DGC patient-derived xenograft (PDX) models that recapitulate the primary cancer. Cells in PDX model GAGA6 with FGFR2 amplification were sensitive to AZD4547, a potent FGFR inhibitor that is being clinically evaluated for FGFR-aberrant cancer types. Intermittent in vivo treatment of GAGA6 tumors with AZD4547 gave rise to PDX tumors with acquired resistance to AZD4547, GAGA6-R. Surprisingly, there were no mutations in the FGFR2 gene in GAGA6-R, negating gatekeeper mutations as a mechanism of drug resistance. Phosphorylation of FGFR2 and downstream signaling molecules AKT/PKB and MAPK/ERK remained inhibited by AZD4547. Further analysis of signaling pathways identified AKT-independent phosphorylation and inhibition of GSK3β as a mechanism of drug resistance in GAGA6-R cells. Treatment of GAGA6-R cells with protein kinase C (PKC) inhibitor H7 in combination with AZD4547 led to dephosphorylation and activation of GSK3β with concomitant downregulation of MCL-1 and BCL-XL. Combined treatment with AZD4547 and H7 in vitro synergistically enhanced cell death in GAGA6-R but not GAGA6 cells. Furthermore, midostaurin, a multikinase inhibitor with PKC-inhibiting activity, in part reversed resistance of GAGA6-R tumor to AZD4547 in vivo. Our results suggest that upon challenge with FGFR inhibitors, FGFR2-amplified tumors that are highly dependent on FGFR2 signaling for survival rapidly develop resistance by switching to a PKC-mediated inhibition of GSK3β to gain a survival advantage. Mol Cancer Ther; 17(1); 1–11. ©2017 AACR.

Introduction

Diffuse-type gastric cancer (DGC) is clinically and histologically distinct from intestinal-type gastric cancer (IGC; ref. 1), and is often characterized by signet ring cell histology (2), as well as rapid infiltration into the stomach wall and frequent metastasis to the lymph nodes and peritoneum (3–5). These factors contribute to a worse prognosis and lower 5-year survival rates than IGC (6–10). Currently, there are no molecular or genetic markers which distinguish gastric tumors, and targeted therapies are urgently needed for effective treatment of this disease.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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against pan-FGFR inhibitor AZD4547 in order to further explore other potential mechanisms of acquired TKI resistance in gastric cancer. We found that drug-resistant cells were able to survive the inhibition of MAPK and AKT pathways by AZD4547, via protein kinase C (PKC)–mediated phosphorylation of GSK3β in GAGA6-R–resistant cells, resulting in inhibition of GSK3β and maintenance of prosurvival proteins MCL-1 and BCL-XL. PKC and FGFR inhibitors in combination synergistically enhanced cell death in GAGA6-R but not GAGA6 cells. Midostaurin, a multikinase inhibitor with PKC-inhibiting activity, in part reversed resistance of GAGA6-R tumor to AZD4547 in vitro. Our findings suggest that FGFR inhibitor resistance may be overcome through concurrent inhibition of PKC signaling events.

**Materials and Methods**

**Study approval**

All patient samples were collected with informed patient consent from National University Hospital Singapore according to the National Healthcare Group Domain Specific Review Board guidelines. All animal experiments were conducted with the approval of Institutional Animal Care and Use Committee in the National University of Singapore (NUS). Clinicopathologic details of patient samples are included in Supplementary Table S1.

**Isolation and purification of cells from peritoneal ascites**

Ascites fluid was collected in sterile 2L Nalgene bottles containing 1 mmol/L EDTA/PBS, before transferring to 500 mL centrifuge bottles for centrifugation at 7,000 g for 15 minutes at 4°C. Supernatant was discarded, and pellets were resuspended in 1 mmol/L EDTA/PBS before layering onto Ficoll-Paque (GE Healthcare) for red blood cell removal at 400 x g for 30 minutes at 4°C. Mononuclear cell fractions were extracted and washed twice with PBS before depletion of hematopoietic cells using CD45 and glycoporphin A antibody–conjugated beads (GlyA; Milteny Biotec). CD45⁺/GlyA-depleted cells were washed with PBS before enumeration with trypan blue using a hemocytometer. Dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec).

**PDX models**

NOD/SCID/IL2Rγ⁻ (NSG) mice were bred in specific pathogen-free mouse facilities in NUS under Material Transfer Agreement (MTA) from Jackson Laboratories. Six- to 8-week-old female mice were used for xenograft transplantation and anesthetized with isoflurane before injection. Note that 1 to 3 x 10⁷ cells were resuspended in Matrigel (BD Biosciences; 2:1 with sterile HBSS) in a final volume of 200 µL per injection and administered subcutaneously. For intraperitoneal xenografts, at least 5 x 10⁵ cells in 300 µL sterile PBS were injected. Mice were monitored for tumor formation and euthanized when tumors were 1.5 cm in diameter. Subcutaneous tumors were aseptically excised and dissociated for propagation, whereas intraperitoneal ascites was drawn out using a sterile syringe and needle and Ficoll-purified before propagation. GAGA6-R AZD4547-resistant PDX model was derived by intermittent drug treatment over 14 weeks, specifically, 4 weeks of AZD4547 treatment (12.5 mg/kg thrice/week), during which tumor growth was inhibited, followed by 3 weeks of withdrawal, and another 7 weeks of AZD4547 treatment, after which tumors gained resistance and were passaged and serially transplanted subcutaneously into NSG mice, which were administered with intraperitoneal AZD4547 for the duration of tumor growth in the mouse.

**In vivo drug treatments**

PDXs GAGA1, GAGA3, GAGA6, and GAGA6-R were engrafted subcutaneously into NSG mice, and drug treatment commenced when tumors were approximately 0.5 cm at the smallest measurement. The following drugs were purchased from Selleck Chemicals. AZD4547 was administered intraperitoneally at a dose of 12.5 mg/kg thrice a week. Cisplatin was administered intraperitoneally at a dose of 2.3 mg/kg once a week. Midostaurin was administered by oral gavage at a dose of 100 mg/kg daily. Mouse weight and tumor measurements were taken twice weekly. Tumor volume was calculated according to the formula 0.5 (length x width²). Tumor growth inhibition (TGI) was expressed as a percentage as follows: TGI (%) = (TGmean Control – TGmean drug/TGmean control) x 100, where mean tumor growth (TGmean) is the difference between the mean tumor volume at the end of treatment and the mean pretreatment tumor volume for individual groups. Vehicle controls were DMSO for both AZD4547 and midostaurin and saline for cisplatin.

**Ex vivo cell culture from PDX tumors**

All cultures were maintained in humidified 37°C incubators supplemented with 5% CO₂. Primary cell cultures were established from xenograft tumors by plating finely minced tissue pieces into DMEM supplemented with 10% FBS. After 48 hours, adherent cells were trypsinized with TryPLE (Life Technologies) and replated. Subculturing was performed until fibroblasts were depleted from the culture. The remaining cells were verified to be EpCAM⁺ by flow cytometric analysis. GAGA6-R ex vivo–cultured cells were obtained from minced GAGA6-R PDX tumors as described above, whereupon GAGA6-R cells were grown without AZD4547 in the culture medium, whereas GAGA6-R⁺ cells were supplemented with 1 mmol/L AZD4547 in the culture medium. All experiments were conducted on cell lines between passages 10 and 30. GAGA6 and GAGA6-R cell lines were authenticated to be unique primary cell lines by STR profiling (9-marker test) and certified to be mycoplasma-free by PCR (June 2017; Idexx Bioresearch).

**Exome sequencing and analysis**

Exome sequencing was performed on tumor cell DNA from patient ascites and normalized to DNA from matched blood samples from the same patient. Exome capture was done using SureSelect Human All Exon V5 + UTR kit (Agilent Technologies) on a HiSeq sequencer (Illumina) using 76- and 90-bp paired-end reads. Image analysis and base calls were performed using Illumina pipeline with default settings. Burrows-Wheeler Aligner software was used for human reference genome (hg19) alignment. SAMtools was used to remove PCR duplicates. Genome Analyzer Toolkit (GATK) was used to identify single-nucleotide variants. Databases dbSNP135 and 1000 Genomes Project were used to remove germline variants. Somatic mutations were detected by subtracting sequence variants of normal exomes from tumor exomes. Nonsynonymous mutations and splice-site mutations were analyzed and validated using Sanger capillary sequencing. Copy-number analysis was performed with Varscan 2.0 [http://varscan.sourceforge.net/; ref. 20] using exome reads as input files.
FISH

FGFR2 FISH was performed on Formalin-fixed paraffin-embedded (FFPE) sections after deparaffinization, pretreatment, and protein digestion. FGFR2 and centromere 10 (CEN10) probes were mixed and hybridized onto sections overnight. Nuclei were stained with DAPI and imaged on a fluorescence microscope under 100x objective. Sixty nuclei per sample were scored, and FGFR2 was considered amplified when the signal ratio of FGFR2/CEN10 probes was greater than or equal to 2. Probes were obtained from SureFish, Agilent Technologies, Inc., or ZytoVision.

Flow cytometric analysis

For surface and intracellular staining, 1 x 10^5 and 3 x 10^5 cells per tube were used, respectively. For cells isolated from clinical gastric cancer samples, CD45^+ blood cells and GlyA^- erythroid precursors and EpCAM^+ cells were gated accordingly for analysis. Cells were analyzed on a BD LSRII flow cytometer (BD Biosciences). Antibodies used were as follows: EpCAM-APC and IgG1-APC (130-091-254 and 130-092-214; Miltenyi Biotec), CD45-FITC and IgG1-FITC (304006 and 400110; Biolegend), and GlyA-FITC (11-9987-82; ebioscience).

Drug inhibitors and Annexin V assay

AZD4547, Sunitinib, and GS3KB inhibitor 1-azakenpaullone (1-AKP; ref. 21) were purchased from Selleck Chemicals, and H7 (22) was purchased from Abcam. GAGA6, GAGA6-R^+, and GAGA6-R PDx tumor cells were cultured in vitro and treated with various inhibitors (AZD4547, 1-AKP, and H7) for 48 hours before trypsinization. Cells were washed and stained with Annexin V-APC according to the manufacturer’s protocol (640941; Biolegend) and analyzed on a BD LSRII (BD Biosciences) flow cytometer for viable (Annexin V-negative) and apoptotic (Annexin V-positive) cells.

siRNA knockdown assays

ON-TARGETplus human FGFR2 siRNAs (LQ-003132-00-0005) and nontargeting control siRNAs (D-001810-01-05) were purchased from GE Dharmacon, diluted in siRNA buffer (300 mmol/L KCl, 30 mmol/L HEPES-pH 7.5, 1 mmol/L MgCl$_2$), and transfected into GAGA6 or GAGA6-R ex vivo–cultured cells using JetPrime transfection reagent (114-15; Polyplus-transfection), according to the manufacturer’s protocol.

Immunoblotting

Total protein was extracted by cell lysis for 15 minutes on ice in buffer containing 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 1% Triton X-100, and 1x complete protease inhibitor cocktail (Roche) before collection of lysate supernatant by centrifugation at 14,000 g at 4°C. Equal amounts of protein (50 µg) as quantitated by Bradford assay were resolved by 10% SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes (Whatman), blocked in 5% milk-TBST, and probed with relevant antibodies. Primary antibodies used were purchased from Cell Signaling Technology unless otherwise indicated: p-FGFR (Y653/654), p-ERK (S473), AKT, p-HER2, HER2, p-EGFR, EGFR, p-PKCα/β (T638/641), p-PC4 (S660), p-PCK8 (T505), p-PC4 (S1219), PKCδ (S1978; Sigma). Membranes were incubated with horseradish peroxidase-conjugated antibodies against mouse or rabbit IgG (Amersham) and detected by chemiluminescence (Immobilon-P; Millipore) signals on Cl-Xposure film (Thermo Scientific).

GS3KB-S9A transfection

GS3KB-S9A cDNA constructs were purchased from Addgene and transfected into GAGA6 or GAGA6-R cells using JetPrime transfection reagent (114-15; Polyplus-transfection).

Histopathology

Tissue samples were fixed in 10% neutral-buffered formalin (Sigma-Aldrich), processed, and stained by Department of Pathology, National University Hospital, Singapore. Routine hematoxylin and cosin (H&E) and Periodic acid–Schiff staining with diastase predigestion was performed on all sections and evaluated by a pathologist.

Apoptosis assay by TUNEL staining

For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining on cryosections, xenograft tumors were excised and 10 mm x 1 mm pieces were fixed in 10% formalin for 6 to 8 hours before transferring to 25% sucrose overnight. Tissues were then embedded in optimal cutting temperature compound (Sakura Finetek) and frozen in liquid nitrogen before sectioning at 5 µm thickness (Leica cryostat CM3050s) and mounting onto glass slides (VWR SuperFrost Plus) for TUNEL staining according to the manufacturer’s protocol (Ab66110; Abcam). Stained slides were visualized using a Nikon A1-R confocal system. For TUNEL staining by flow cytometry, single cells were purified from minced xenograft tumors as described previously (23) and stained according to the manufacturer’s protocol (Ab66108; Abcam).

PCR and real-time qPCR analysis

Note that 400 ng total RNA isolated with the RNeasy Micro Kit (Qiagen) was reverse-transcribed using the Maxima First Strand cDNA synthesis Kit (Fermentas). qRT-PCR analysis was performed using the Maxima SYBR Green/Rox qPCR master mix (Fermentas) using the following parameters: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. 18S rRNA was used as an internal control. Relative expression among samples was calculated using the comparative quantitation method (Rotor-Gene Q software). Gene-specific primers (Integrated DNA Technologies) used are listed as follows.

Sanger sequencing

DNA was isolated from PDX tumors and reverse-transcribed as described above for qRT-PCR analysis. FGFR2 coding region sequence analysis was performed by the Sanger sequencing method using BigDye Terminator chemistry on ABI sequencers.
The determination of combination index for drug treatments

Drug combination regimens were designed according to recommended methodology by Chou (24). AZD4547 (10–500 nmol/L) and H7 (2–100 μmol/L) drug combination ratios were fixed at 1:200. CompuSyn software was used to determine synergy between drugs in combination (http://www.combinesyn.com/). Combination index (CI) values were generated by the software which assists in determination of drug combination doses. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, or antagonism, respectively.

Statistical analysis

All error bars represent the mean ± SD. Unpaired two-tailed Student t tests or two-way ANOVA was performed using GraphPad Prism (GraphPad Software). For all statistical tests, P < 0.05 was considered to be significant.

Results

Development and validation of robust DGC PDX models

Establishing PDX models for DGC has posed a significant challenge for us and others in the field (25, 26). To overcome the difficulty in obtaining primary tumors of sufficient tumor cellularity from DGC tissues, we collected peritoneal ascites fluid from DGC patients (Supplementary Table S1) and depleted GlyA+ red blood cells and CD45+ hematopoietic cells. As EpCAM is a tumor-associated antigen that is highly expressed in gastric cancer (23, 27), we reasoned that EpCAM+ cells found in ascites fluid of gastric cancer patients would likely be exfoliated tumor cells from the primary tumor site. EpCAM+ cells were observed as viable, single cells and were visibly larger in size than CD45+ cells as shown in cytospin preparations (Fig. 1A). Following depletion of blood cells, we obtained highly purified EpCAM+ tumor cells (>99%; Fig. 1B) which were subjected to downstream analyses such as establishment of PDXs and exome sequencing.

We evaluated the tumor formation ability of EpCAM+ cells by subcutaneously transplanting cells isolated from GAGA1, GAGA3, and GAGA6 patients into immune-deficient NOD/SCID/IL2Rγ− (NSG) mice, with the exception of GAGA2, which did not yield enough cells for xenotransplantation. All injected cells formed PDXs within 3 months that could be serially passaged in mice. H&E staining of these subcutaneous xenografts demonstrated high fidelity to the primary tumors (Supplementary Fig. S1A).

Gene alterations revealed by exome sequencing of primary and xenograft tumors

Exome sequencing analyses revealed a high degree of concordance in mutations between tumor cells from the primary ascites and PDX for GAGA1 and GAGA6, which show 98% and 92% concordance, respectively. GAGA3 subcutaneous (GAGA3-SC) and intraperitoneal (GAGA3-IP) PDXs had concordances of 54% and 75%, respectively, suggesting tumor heterogeneity. Further analysis of mutations and gene alterations revealed FGR2 gene amplification in GAGA patients 2, 3, and 6 (Fig. 1C; Supplementary Fig. S1B; Supplementary Tables S2 and S3). Notably, the presence of at least two clonal populations in GAGA3 was evident from the observation that tumor cells from primary ascites showed both FGR2 and KRAS amplification, whereas GAGA3-SC PDX tumors preferentially selected for FGR2 amplification, whereas GAGA3-IP PDX tumors had KRAS but not FGR2 amplification (Fig. 1C).

FGR2 expression in FGR2-amplified PDX models of DGC

As there are ongoing clinical investigations of FGR/FGR-targeted agents in advanced malignancies including gastric cancer, we decided to test our FGR2-amplified PDX models for FGR inhibitor response in preclinical studies. We confirmed the status of FGR2 gene amplification in the GAGA PDX models by FISH. Consistent with exome sequencing data (Fig. 1C), GAGA3 and GAGA6 PDX tumors were FGR2-amplified, whereas GAGA1 PDX cells were not (Fig. 1D). Correspondingly, FGR2 transcript levels were 100-fold higher in GAGA3 and GAGA6 PDX tumor cells compared with GAGA1, as well as with xenograft cells from previously established IGC xenograft models (Fig. 1E; ref. 23). FGR2 protein expression levels were also found to correlate with genetic alteration and transcript levels; FGR2 was highly expressed in GAGA3 and GAGA6, but not GAGA1 cells (Fig. 1F).

FGR2-amplified DGC cells are dependent on FGR2 signaling for survival

As a proof of concept, we employed our PDX models to study the sensitivity of FGR2-amplified versus nonamplified tumors toward AZD4547, a small-molecule FGR inhibitor currently in clinical trials for patients with FGR1/2-amplified breast, lung, or gastric cancer (NCT01795768). FGR2-amplified GAGA3 (Supplementary Fig. S2A) and GAGA6 PDX tumors were highly sensitive to AZD4547 treatment and showed significant TGI (100%; Fig. 2A) despite amplification of KRAS and MYC, respectively, suggesting that FGR2 is a dominant driver of tumor growth. In contrast, FGR2 nonamplified GAGA1 tumors did not respond to AZD4547 treatment, and no significant difference in tumor size was observed between AZD4547-treated or vehicle controls (TGI = 14%; Fig. 2A). Regardless of FGR2 amplification status, both GAGA6 and GAGA1 tumors responded poorly to treatment with cisplatin, an existing standard-of-care chemotherapy agent, with no significant regression in tumor size (Fig. 2B).

Drug-treated xenograft tumors were analyzed for apoptotic cells using a TUNEL assay by fluorescence (Supplementary Fig. S2B). AZD4547 treatment resulted in a significant increase in TUNEL+ cells in GAGA6 tumors compared with vehicle-treated cells (Fig. 2C; Supplementary Fig. S2B). In contrast, cisplatin treatment of either GAGA6 or GAGA1 tumors did not result in significant cell death (Supplementary Fig. S2B). Collectively, these data show that FGR2 is a dominant driver of tumor growth in spite of MYC and KRAS coamplification status, and is a druggable target using FGR2 inhibitors.

To evaluate downstream signaling molecules upon drug treatment, GAGA6 PDX mice were treated in time-course
experiments with a single dose of AZD4547 followed by immunoblot analyses of protein extracts from GAGA6 PDX tumors. AZD4547 showed profound and sustained inhibition of FGFR phosphorylation by 30 minutes, and after 2 hours, phospho-FGFR2 was undetectable (Fig. 2D). The drug did not affect levels of total FGFR2 proteins indicating its primary effect on FGFR2 kinase activity. AZD4547 also effectively reduced phosphorylation of FGFR2 downstream targets as levels of phospho-AKT and phospho-ERK were also downregulated (Fig. 2D), confirming pathway modulation in vivo.

AZD4547 resistance in cell culture–derived (GAGA6-R’) and PDX (GAGA6-R) models

Despite the initial favorable response to chemotherapy, most tumors invariably develop drug resistance. Current efforts to understand FGFR inhibitor resistance have focused on in vitro cell line modeling, and gatekeeper mutations in the kinase domain of many FGFRs have been reported as a mechanism for acquired drug resistance; however, it is not known whether these findings are clinically relevant (28, 29).

We established an ex vivo GAGA6-R’ drug-resistant model by subjecting GAGA6 ex vivo–cultured tumor cells to prolonged AZD4547 treatment (Fig. 3A). Gatekeeper mutations which impair drug binding in the ATP-binding pocket of FGFR were previously identified as a mechanism of resistance in cell line–based assays (29, 30). Consistent with this, we observed the emergence of a FGFR2 V565F gatekeeper mutation in GAGA6-R’-resistant cells (Fig. 3B) that was drug nonresponsive as FGFR2 phosphorylation could not be inhibited by AZD4547 treatment (Fig. 3C).
In order to elucidate other potential resistance mechanisms, we developed an in vivo FGFR inhibitor–resistant PDX model. We subjected GAGA6 tumor–bearing mice to intermittent AZD4547 treatment over a period of 14 weeks without passing, after which the tumors developed resistance to AZD4547 and were able to grow in the presence of AZD4547 (Fig. 3D). Drug resistance was achieved at week 10, when tumors continued to grow despite AZD4547 treatment. These tumors were excised from the mice and passaged twice in vivo with continuous AZD4547 treatment for a total of 28 weeks (Fig. 3D). FGFR2 amplification in GAGA6-R PDX tumors was confirmed by FISH (Fig. 3E), demonstrating the emergence of acquired resistance rather than outgrowth of cancer subclones that did not harbor FGFR2 amplification. GAGA6-R PDX tumors were also subjected to exome sequencing, and no gatekeeper mutations were detected in any FGFR kinase domains. We further confirmed this by Sanger sequencing of FGFR2 kinase domain (Fig. 3B). No additional mutations were detected in signaling molecules downstream of FGFR2, such as AKT, ERK, and GSK3. We also did not detect amplifications of other receptor tyrosine kinase (RTK) pathway receptors such as EGFR and HER2. The lack of gatekeeper mutations in the FGFR2 gene was also observed in GAGA3-R, an in vivo resistance model developed similarly to GAGA6-R (Supplementary Fig. S2C and S2D). Similarly, GAGA3-R cells are resistant to the killing effects of AZD4547, but continued to express high levels of FGFR2 (Supplementary Fig. S2E).

FGFR inhibitor resistance in GAGA6-R cells occurs independently of MAPK or AKT pathways

To determine the requirement of FGFR2 for tumor survival, we performed siRNA-mediated knockdown of FGFR2 in GAGA6 and GAGA6-R ex vivo tumor cells (Fig. 4A, top). Knockdown of FGFR2 substantially reduced the protein level in both GAGA6 and GAGA6-R cells; cell death was observed in GAGA6 cells, which was, however, markedly less in GAGA6-R cells (Fig. 4A, bottom), suggesting the emergence of FGFR2 independence. To determine if FGFR2 had been rendered unresponsive to inhibition by AZD4547, GAGA6-R tumors were treated in vivo with AZD4547 and assayed for activation of FGFR2 as well as downstream signaling proteins. Consistent with a lack of gatekeeper mutations in the kinase domain of FGFR2 in GAGA6-R cells, phosphorylation of FGFR was effectively inhibited by AZD4547 treatment as early as 30 minutes after administration of the drug (Fig. 4B). Furthermore, inhibition of FGFR phosphorylation resulted in corresponding inhibition of AKT and ERK phosphorylation. We also did not observe upregulation or activation by phosphorylation of HER2 and EGFR in GAGA6-R PDX tumors, whether in the presence or absence of FGFR2 inhibition (Supplementary Fig. S3A).
In contrast to parental GAGA6 tumor cells where the phosphorylation of GSK3β, a downstream target of AKT, was inhibited by AZD4547 (Fig. 2D), GSK3β remained constitutively phosphorylated in GAGA6-R cells (Fig. 4B), suggesting that the mechanism of drug resistance was mediated by sustained phosphorylation of GSK3β via an AKT-independent pathway. In addition, treatment of GAGA6-R cells with sunitinib, a multi-receptor TKI that exerts antitumor activity through dephosphorylation of AKT (31, 32), did not lead to dephosphorylation of GSK3β and subsequent cell death (Supplementary Fig. S3B and S3C), further substantiating that in GAGA6-R cells GSK3β phosphorylation occurs independently of AKT or other RTK pathways.

Constitutive phosphorylation of GSK3β mediates drug resistance to FGFR inhibitor

GSK3β is a multifunctional serine/threonine kinase whose activity is inhibited by phosphorylation of its serine 9 residue (33). It is a critical downstream element of the PI3K/AKT pathway, and phosphorylation of GSK3β by AKT promotes cell growth and survival; hence, its dysregulation has been implicated in tumorigenesis. However, the role of GSK3β in tumorigenesis and cancer progression remains controversial; it either promotes or inhibits tumor growth in different tumor models (33). Our results suggest that an inactive GSK3β that is phosphorylated at serine 9 is important for tumor cell survival (Fig. 4C). In GAGA6 parental cells, AZD4547 inhibits phosphorylation of FGFR2 and downstream phosphorylation of GSK3β at serine 9, thus activating the protein. Signaling from active GSK3β in turn leads to cell death. However, in GAGA6-R drug-resistant cells, GSK3β remains phosphorylated at serine 9 (inactivated state) in the presence of AZD4547. To determine if inactivation of GSK3β conferred resistance to AZD4547 in GAGA6-R cells, we treated both GAGA6 parental and GAGA6-R cells ex vivo with the GSK3β inhibitor 1-azakenpaullone (1-AKP). Survival of both GAGA6 and GAGA6-R cells was unaffected by 1-AKP treatment alone up to 10 μmol/L (Supplementary Fig. S3D). However, GAGA6 cells treated with a combination of 1-AKP (at 100 nmol/L) and AZD4547 exhibited increased resistance to AZD4547 compared with control cells treated with AZD4547 alone (Fig. 4D), suggesting that inhibition of GSK3β in GAGA6 cells confers resistance to AZD4547. Unsurprisingly, 1-AKP did not alter the sensitivity of GAGA6-R cells to AZD4547 (Fig. 4D). In reciprocal experiments, because a direct activator of GSK3β is not available, we introduced a constitutively active GSK3β-S9A mutant into GAGA6-R cells and observed that they were sensitized to AZD4547 treatment (Fig. 4E). Taken together, these results demonstrate that constitutive phosphorylation and hence sustained inhibition of GSK3β in GAGA6-R cells mediate resistance to AZD4547 treatment and provide a mechanism for cell survival in spite of FGFR inhibition.

Phosphorylation of GSK3β by PKC is important for cell survival by maintaining levels of MCL-1 and BCL-XL

GSK3β is a known substrate of PKC (34). To investigate the involvement of PKC pathway in GSK3β signaling in GAGA6-R cells, we treated the cells with H7, a potent PKC inhibitor. We observed inhibition of phospho-PKCγ and phospho-GSK3β in both GAGA6 parental and GAGA6-R cells (Fig. 4F). Phosphorylation of other PKC isoforms remained unaltered with H7 treatment (Supplementary Fig. S4). Treatment of GAGA6-R cells with PKC inhibitor H7 in combination with AZD4547 led to dephosphorylation and hence activation of GSK3β, with
Figure 4.
AKT-independent GSK3β-mediated drug resistance in GAGA6-R tumor cells. A, Ex vivo-derived cells were transfected with Dharmacon On-target plus human FGFR2 siRNA pool or nontargeting siRNA pool (4 siRNA duplexes each). Cell lysates of the transfected cells were analyzed by immunoblot analysis for phosphorylated FGFR and FGFR2. Increased numbers of Annexin V-positive cells were observed following knockdown of FGFR2. B, Immunoblot analysis of total protein extracts of GAGA6-R PDX tumors following treatment with AZD4547 or vehicle control. Tumors were harvested at indicated time points for immunoblotting analyses using antibodies against indicated proteins with β-actin as a loading control. C, Working hypothesis of GSK3β-mediated drug resistance: In GAGA6 cells, AZD4547 inhibits FGFR2 phosphorylation, thereby inhibiting downstream phosphorylation of GSK3β (activated state) which leads to cell death. In GAGA6-R cells, GSK3β remains phosphorylated (inactivated state) in the presence of AZD4547. D, Inhibition of GSK3β kinase activity by 1-AKP (100 nmol/L) in the presence or absence of AZD4547. Cells were drug-treated for 48 hours. Immunoblot analysis of total protein extracts following indicated treatments. Dose-response curve shows sensitivity of GAGA6 and GAGA6-R ex vivo–cultured cells to 1-AKP (100 nmol/L) with increasing concentration of AZD4547. Percentage of live cells after drug treatment was determined by Annexin V staining. E, Overexpression of constitutively active GSK3β-S9A in GAGA6-R tumor cells and dose-dependent response to AZD4547 treatment measured by Annexin V staining. F, Immunoblot analysis of total protein extracts of GAGA6 and GAGA6-R cells following treatment with AZD4547, H7, or vehicle control. G, Annexin V cell death assay in GAGA6 and GAGA6-R cells following treatment with AZD4547, H7, or in combination. H, CI and fractional inhibition values of AZD4547 and H7 drug treatments, computed by CompuSyn software. CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively. I, Effect of midostaurin (100 mg/kg daily), AZD4547 (125 mg/kg thrice/week), and combination treatment on GAGA6-R xenograft tumors for 12 days. N = 6 for all treatment groups. Statistical analyses were performed using two-way ANOVA between two groups.
concomitant downregulation of prosurvival proteins MCL-1 and BCL-XL (Fig. 4F). We performed Annexin V cell death assays and observed that combined treatment of GAGA6-R cells with AZD4547 and H7 resulted in increased apoptosis (25%), compared with marginal apoptosis when the cells were treated with either AZD4547 (2%) or H7 (7%) alone (Fig. 4G). In contrast, combined AZD4547 and H7 treatment of GAGA6 parental cells did not induce a higher proportion of apoptotic cell death compared with treatment with either AZD4547 or H7 alone (Fig. 4G). To assess if AZD4547 could act in synergy with H7, we performed drug combination experiments according to the method of Chou (24) with varying drug doses at fixed drug ratios, and computed the fractional inhibition effect of individual drugs as well as the CI of both drugs combined, using CompuSyn software (Fig. 4H). Combination treatment with AZD4547 and H7 synergistically enhanced cell death in GAGA6-R cells at all three drug doses, as indicated by their CI of less than 1. In contrast, no drug synergy was observed in GAGA6 cells for all three drug doses (Fig. 4H), suggesting acquisition of PKC-dependent survival in GAGA6-R cells.

**In vivo inhibition of PKC increases sensitivity of GAGA6-R tumors to AZD4547**

We treated GAGA6-R tumors in vivo with midostaurin, an FDA-approved multikinase inhibitor for leukemia treatment with high potency against PKCζ/δ/γ (35). Midostaurin alone significantly inhibited growth of GAGA6-R tumor growth (Fig. 4I; 75% TGI, \( P < 0.0001 \)), which was further enhanced when midostaurin was combined with AZD4547 (Fig. 4I; 95% TGI, \( P < 0.0001 \)). Importantly, AZD4547 alone was unable to inhibit the growth of GAGA6-R tumors (Fig. 4I; 13% TGI). These results clearly demonstrate that midostaurin increases sensitivity of GAGA6-R tumors to AZD4547.

**Discussion**

Targeted therapies for gastric cancer are still in the early phases of clinical evaluation and development, partly due to a lack of known effective molecular targets (36). Targeting FGFR2 is an attractive therapeutic option for DGC, as FGFR2 amplification is found in approximately 10% of all gastric tumors, and high FGFR2 expression has been correlated with tumor progression and poor survival in DGC (37). A recent clinical trial has demonstrated that gastric cancers with high-level FGFR2 amplification have a high response rate to AZD4547 (15), and several clinical and preclinical studies have also shown that FGFR amplification predicts sensitivity to FGFR inhibitors such as BGJ398 and AZD4547 (38–40). Concurrent with these studies, there has been much focus on pre-empting acquired clinical resistance to FGFR inhibitors (30, 41, 42). In a recent study, the acquisition of a gatekeeper mutation, p.V564F, in FGFR2 was found to confer resistance in three cholangiocarcinoma patients with acquired clinical resistance to BGJ398 (43). This study highlights the first clinical occurrence of the FGFR2 V564F gatekeeper mutation as a mechanism of acquired resistance, which had been previously investigated in preclinical studies (28, 29). In gastric cancer, clinical resistance to FGFR inhibitors has not been well reported, and our understanding of acquired resistance mechanisms is limited. Consequently, we sought to identify potential mechanisms of acquired resistance to FGFR inhibitors using in vivo patient-derived DGC models.

In contrast to cell line–based studies (29), GAGA6 PDX tumors that were under continuous AZD4547 treatment in vivo did not develop gatekeeper mutations in the ATP-binding pocket of FGFR2. The lack of gatekeeper mutations in AZD4547-resistant cells was also observed in another FGFR2-amplified PDX, GAGA3, when subjected to AZD4547 selection in vivo (Supplementary Fig. S2D), suggesting that the factors which contribute to drug resistance in gastric cancer may depend on growth conditions in vitro and in vivo. In addition, gastric cancer patients with disease progression upon AZD4547 treatment did not develop gatekeeper mutations (44). Taken together, in vivo modeling of drug resistance using PDX models may reveal resistance mechanisms that are clinically relevant for gastric cancer.

In our study, we explored several mechanisms of drug resistance. GAGA6-R cells remained FGFR2-amplified as analyzed by FISH (Fig. 2F), demonstrating that secondary resistance did not arise due to clonal selection of FGFR2-nonamplified cells. Activation of compensatory downstream signaling pathways through redundant kinases has also been shown to mediate drug resistance (45). We investigated alternate activation of two receptor tyrosine kinases, EGFR and HER2, and found that both were expressed at low levels in both GAGA6 and GAGA6-R tumors and were not significantly upregulated or phosphorylated in response to AZD4547 (Supplementary Fig. S3A).

Multitarget kinase inhibitors such as sunitinib were developed as a strategy to overcome cancer relapse in cancer patients who developed drug resistance due to the activation of alternative RTK pathways (46). We employed sunitinib as a chemical probe to determine if any of its RTK targets might contribute to AZD4547 resistance in GAGA6-R tumor cells. Our data demonstrated that GAGA6-R cells remained viable upon sunitinib treatment, whether in the presence or absence of AZD4547 (Supplementary Fig. S3C), thereby precluding the involvement of sunitinib target receptors VEGFR, PDGFR, FLT-3, and c-Kit.

Most intriguingly, in spite of the ability of GAGA6-R tumors to grow in the presence of AZD4547, we observed inhibition of FGFR and AKT phosphorylation while GSK3β remained constitutively phosphorylated, pointing to an unusual disconnect between AKT and GSK3β (Fig. 4B). These findings prompted us to examine alternate GSK3β-activating pathways that may bypass AKT signaling. We found that in GAGA6-R cells, inhibition of PKC led to dephosphorylation of GSK3β as well as downregulation of prosurvival proteins MCL-1 and BCL-XL, resulting in increased cell death (Fig. 4F and G). Furthermore, the combined effect of PKC inhibitor H7 and AZD4547 treatment in vitro synergistically enhanced cell death in GAGA6-R cells but not in GAGA6 parental cells. Concomitant suppression of PKC and FGFR in vivo using midostaurin and AZD4547 markedly inhibited the growth of GAGA6-R tumors (Fig. 4I), suggesting that when FGFR2 signaling is suppressed in GAGA6-R cells, the PKC pathway functions as an alternate signaling pathway to maintain phosphorylation and inhibition of GSK3β to confer a survival advantage through its downstream prosurvival targets. Rewiring of signaling pathways in AZD4547-resistant tumors has been reported which involved alternate activation of the mTOR pathway, acquisition of KRAS mutation, and various resistant genotypes in their drug-resistant PDX models generated from patients who had durable clinical response to AZD4547 (47). Our findings demonstrate for the first time a PKC-dependent rewiring mechanism to confer resistance to AZD4547.
In light of our findings and data from the field, it is apparent that acquired resistance to RTK inhibitors can be mediated through myriad alternate signaling pathways, including “hijacking” of well-characterized canonical pathways. Prevention of clinical resistance to AZD4547 in patients with FGFR2 amplification may be achieved by combination therapies using nonoverlapping drug resistance profiles, for instance a multikinase inhibitor such as midostaurin in combination with a specific FGFR inhibitor such as AZD4547.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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PKC-Mediated GSK3β Inactivation Confers AZD4547 Resistance

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