Title: Acquired resistance to FGFR inhibitor in diffuse-type gastric cancer through an AKT-independent PKC-mediated phosphorylation of GSK3β

Authors: Wen Min Lau1, Eileen Teng1, Kie Kyon Huang1,2, Jin Wei Tan1, Kakoli Das2, Zhijiang Zang2, Tania Chia1, Ming Teh3, Koji Kono1,4, Wei Peng Yong1,2, Asim Shabbir4, Amy Tay4, Niam Sin Phua4, Patrick Tan1,2, Shing Leng Chan1,*, Jimmy Bok Yan So4.

Affiliations:
1Cancer Science Institute of Singapore, National University of Singapore
2Cancer and Stem Cell Biology Program, Duke-NUS Medical School, Singapore
3Department of Pathology, National University of Singapore
4Department of Surgery, National University of Singapore, National University Hospital
5Department of Haematology-Oncology, National Cancer Institute of Singapore, National University Hospital, Singapore,

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*Corresponding authors:  
Shing Leng Chan, PhD  
Cancer Science Institute of Singapore  
National University of Singapore  
14 Medical Drive, MD6, #12-01, Singapore 117599  
Tel: 65-65167211, Fax: 65-68739664.  
Email: csicsl@nus.edu.sg

Patrick Tan, MD, PhD  
Cancer and Stem Cell Biology Program  
Duke-NUS Medical School  
8 College Road, 169857  
+65-65161783  
Email: gmstanp@duke-nus.edu.sg
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 diffuse type gastric cancer 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 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 multi-kinase 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 signalling for survival rapidly develop resistance by switching to a PKC-mediated inhibition of GSK3β to gain a survival advantage.
INTRODUCTION

Diffuse type gastric cancer (DGC) is clinically and histologically distinct from intestinal type gastric cancer (IGC) (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 intestinal type gastric cancer (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.

Approximately 4-10% of primary gastric cancers harbour fibroblast growth factor receptor 2 (FGFR2) amplification (11, 12), hence there is significant interest in FGFR2 as a therapeutic target. FGFR signalling drives many downstream pathways, including MAPK and AKT pathways which are crucial for cell proliferation, survival and migration (13). Consequently, deregulation of FGFR signalling through gene amplification, chromosomal translocation, or gain-of-function mutations has been shown to initiate cancer and sustain cancer cell proliferation due to enhanced kinase activity and constitutive activation of downstream targets (14). Recent clinical studies have demonstrated that FGFR-amplified tumors are highly sensitive to FGFR inhibition and therefore susceptible to therapeutic targeting using selective small molecule FGFR inhibitors such as AZD4547 and BGJ398 (15, 16). However, despite initial sensitivity to FGFR inhibitors in the clinic, patients rapidly develop resistance against these agents. Known mechanisms of acquired drug resistance to tyrosine kinase inhibitors (TKIs) include secondary mutations in drug-binding pockets (gatekeeper mutations), alterations in upstream or downstream effectors, or bypass mechanisms involving activation of parallel or cross-talk signalling pathways (17). Several preclinical and clinical studies have provided valuable insight into FGFR inhibitor resistance mechanisms in gastric cancer (18, 19); however there is still much to be understood in order to find effective targeted therapies this disease.

In this study we generated patient derived xenograft models for DGC and developed an in vivo drug resistance model 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 PKC-mediated phosphorylation of GSK3β in GAGA6-R resistant cells resulting in inhibition of GSK3β and maintenance of pro-survival
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 multi-kinase inhibitor with PKC inhibiting activity in part reversed resistance of GAGA6-R tumor to AZD4547 \textit{in vivo}. Our findings suggest that FGFR inhibitor resistance may be overcome through concurrent inhibition of PKC signalling 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 (IACUC) in the National University of Singapore (NUS). Clinicopathological details of patient samples are included in Supplementary Table 1.

Isolation and purification of cells from peritoneal ascites

Ascites fluid was collected in sterile 2L Nalgene bottles containing 1mM EDTA/PBS, before transferring to 500 mL centrifuge bottles for centrifugation at 7000 g for 15 minutes at 4°C. Supernatant was discarded and pellets were resuspended in 1mM EDTA/PBS before layering onto Ficoll-Paque (GE Healthcare) for red blood cell removal at 400 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 glycophorin A antibody-conjugated beads, (GlyA; Miltenyi). CD45+/GlyA depleted cells were washed with PBS before enumeration with trypan blue using a haemocytometer. Dead cells were removed using the Dead Cell Removal kit (Miltenyi Biotec, USA).

Patient-derived xenograft models

NOD/SCID/IL2Rγ- (NSG) mice were bred in specific pathogen-free mouse facilities in NUS under MTA from Jackson Laboratories. 6 to 8 week old female mice were used for xenograft transplantations and anesthetized with isofluorane before injection. 1-3x10^5 cells were resuspended in Matrigel (BD Biosciences; 2:1 with sterile HBSS) in a final volume of 200µL per injection and administered subcutaneously. For intraperitoneal xenografts, at least 5x10^5 cells in 300 µL sterile PBS were injected. Mice were monitored for tumor formation and euthanized when tumors were 1.5cm in diameter. Subcutaneous tumors were aseptically excised and dissociated for propagation, while 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 (12.5mg/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 intra-peritoneal AZD4547 for the duration of tumor growth in the mouse.

**In vivo drug treatments**

Patient-derived xenografts GAGA1, GAGA3, GAGA6 and GAGA6-R were engrafted subcutaneously into NSG mice, and drug treatment commenced when tumors were approximately 0.5cm at the longest measurement. The following drugs were purchased from Selleck Chemicals (Houston, Texas). AZD4547 was administered intraperitoneally at a dose of 12.5mg/kg thrice a week. Cisplatin was administered intraperitoneally at a dose of 2.3mg/kg once a week. Midostaurin was administered by oral gavage at a dose of 100mg/kg daily. Mouse weight and tumor measurements were taken twice weekly. Tumor volume was calculated according to the formula 0.5(length \times width^2). Tumor growth inhibition was expressed as a percentage as follows: TGI (%) = (TG_{mean\ control} – TG_{mean\ drug} / TG_{mean\ control}) \times 100, where mean tumor growth (TG_{mean}) is the difference between the mean tumor volume at the end of treatment and the mean pre-treatment 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% fetal bovine serum. After 48 hours, adherent cells were trypsinized with TryPLE (Life Technologies) and re-plated. Sub-culturing 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 whilst GAGA6-R+ cells were supplemented with 1mM AZD4547 in the culture medium. All experiments were conducted on cell lines between passages 10-30. 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, Columbia, MO, USA).

**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+UTRkit (Agilent Technology, CA) on a HiSeq sequencer (Illumina) using 76-bp and 90-bp paired-end reads. Image analysis and base calls were performed using Illumina pipeline with default settings. Burrows-Wheeler Aligner (BWA) 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 (SNVs). 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 by Varscan 2.0 (http://varscan.sourceforge.net/ (20)) using exome reads as input files.

**Fluorescence in situ hybridization (FISH)**

*FGFR2* FISH was performed on 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 fluorescent microscope under 100x objective. 60 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. CA, USA or ZytoVision, Germany).

**Flow cytometric analysis**

1x10^5 and 3x10^5 cells per tube were used for direct and indirect staining 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: 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 GSK3β inhibitor 1-azakenpaullone (1-AKP) (21) were purchased from Selleck Chemicals, and H7 (22) was purchased from Abcam. GAGA6, GAGA6- R+ and GAGA6- R PDX tumor
cells were cultured ex vivo and treated with various inhibitors (AZD4547, 1-AKP, H7) for 48 hours before trypsinization. Cells were washed and stained with Annexin V-APC according to 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 non-targeting control siRNAs (D-001810-01-05) were purchased from GE Dharmacon (USA), diluted in siRNA buffer (300mM KCl, 30mM HEPES-pH 7.5, 1mM MgCl₂) and transfected into GAGA6 or GAGA6-R ex vivo cultured cells using JetPrime transfection reagent (114-15, Polyplus-transfection, France), according to manufacturer's protocol.

**Immunoblotting**

Total protein was extracted by cell lysis for 15 minutes on ice in buffer containing 150mM NaCl, 10mM Tris-HCl pH 7.4, 5mM EDTA, 1% Triton X-100 and 1x complete protease inhibitor cocktail (Roche) before collection of lysate supernatant by centrifugation at 14,000g, 4°C. Equal amounts of protein (50µg) 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 (MA, USA) unless otherwise indicated: p-FGFR (Y653/654), p-AKT (S473), AKT, p-ERK, ERK, p-GSK3α/β (S21/9), GSK3α/β, p-HER2, HER2, p-EGFR, EGFR, p-PKCα/β (T638/641), p-PKCβII (S660), p-PKCγ (T505), p-PKCγ (T514), FGFR2 (3F8, Abcam), PKCα (179522, Abcam) and β-actin (A1978, 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).

**GSK3β-S9A transfection**

GSK3β-S9A cDNA constructs were purchased from Addgene (USA) and transfected into GAGA6 or GAGA6-R cells using JetPrime transfection reagent (114-15, Polyplus-transfection, France).
Histopathology

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

Apoptosis assay by TUNEL staining

For TUNEL staining on cryosections, xenograft tumors were excised and 10mm x 1mm pieces were fixed in 10% formalin for 6-8 hours before transferring to 25% sucrose overnight. Tissues were then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and frozen in liquid nitrogen before sectioning at 5µm thickness (Leica cryostat CM3050s) and mounting onto glass slides (VWR SuperFrost Plus, USA) for TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) 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 manufacturer’s protocol (Ab66108, Abcam).

PCR and real-time quantitative PCR (qPCR) analysis

400ng 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, Singapore) used are listed as follows.

18S rRNA Fw/Rv 5′-3′: CCTGCGGGCTTAATTTGACTC/CGCTGAGCCAGTCAGTGTAG;
FGFR2 Fw/Rv 5′-3′: CGTGGAAAAGAACGGCAGTA/TCGGTCACATTGAACAGAGC;
KRAS Fw/Rv 5′-3′: AGACACAAAACAGGCTCAGGA/ATTGTCGGATCTCCCTCACC;
MYC Fw/Rv 5′-3′: CCCTCAACGTTAGCTTACCC/ GAAGGGAGAAGGGTGTGACC.
Sanger sequencing

RNA was isolated from PDX tumours 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 (Applied Biosystems, AIT Biotech Singapore).

Gene specific primers used for analysis are listed:

Fw2/Rv2 5′-3′ gttctcaagcactcggggata/ ggggtggaggtcatgag
Fw3/Rv3 5′-3′ ccaagaacgcaacttcagc/ atgacatagaggcccatcc
Fw4/Rv4 5′-3′ tgggtcagatggagatga/ aaaaagttcctacgggaat
Fw5/Rv5 5′-3′ ttccttcggggtgtaatgtg/ tgcttgaaagtgggtctctg
Fw6/Rv6 5′-3′ tcctcgggtgttaatgtg/ ctctttgttgagagtgaga

Determination of combination index for drug treatments

Drug combination regimens were designed according to recommended methodology by Chou (24). AZD4547 (10-500nM) and H7 (2-100μM) drug combination ratios were fixed at 1:200. CompuSyn software was used to determine synergy between drugs in combination (http://www.combosyn.com/).

Combination index (CI) values were generated by the software which assists in determination of drug combination doses. CI <1, CI = 1, CI >1 represent synergism, additivity, or antagonism, respectively.

Statistical analysis

All error bars represent the mean ± SD. Unpaired two-tailed Student’s t-tests or 2-way ANOVA were performed using GraphPad Prism (GraphPad Software). For all statistical tests, \( P<0.05 \) was considered to be significant.
RESULTS

Development and validation of robust diffuse type gastric cancer PDX models

Establishing PDX models for diffuse type gastric cancer 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 diffuse type gastric cancer patients (Supplementary Table 1) 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 patient derived xenografts (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 xeno-transplantation. All injected cells formed patient derived xenografts (PDXs) within 3 months that could be serially passaged in mice. Haematoxylin and eosin (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 FGFR2 gene amplification in GAGA patients 2, 3 and 6 (Fig. 1C; Supplementary Fig. S1B, Supplementary Tables 2&3). Notably, the presence of at least two clonal populations in GAGA3 was evident from the observation that tumor cells from primary ascites showed
both FGFR2 and KRAS amplification, whereas GAGA3-SC PDX tumors preferentially selected for FGFR2 amplification, while GAGA3-IP PDX tumors had KRAS but not FGFR2 amplification (Fig. 1C).

**FGFR2 expression in FGFR2-amplified PDX models of diffuse gastric cancer**

As there are ongoing clinical investigations of FGF/FGFR-targeted agents in advanced malignancies including gastric cancer, we decided to test our FGFR2-amplified PDX models for FGFR inhibitor response in preclinical studies. We confirmed the status of FGFR2 gene amplification in the GAGA PDX models by fluorescence in situ hybridization (FISH). Consistent with exome sequencing data (Fig. 1C), GAGA3 and GAGA6 PDX tumor cells were FGFR2-amplified while GAGA1 PDX cells were not (Fig. 1D). Correspondingly, FGFR2 transcript levels were 100-fold higher in GAGA3 and GAGA6 PDX tumor cells compared to GAGA1, as well as to xenograft cells from previously established intestinal type gastric cancer xenograft models (Fig. 1E) (23). FGFR2 protein expression levels were also found to correlate with genetic alteration and transcript levels; FGFR2 was highly expressed in GAGA3 and GAGA6, but not GAGA1 cells (Fig. 1F).

**FGFR2-amplified diffuse gastric cancer cells are dependent on FGFR2 signaling for survival**

As a proof-of-concept, we employed our PDX models to study the sensitivity of FGFR2-amplified versus non-amplified tumors towards AZD4547, a small molecule FGFR inhibitor currently in clinical trials for patients with FGFR1/2-amplified breast, lung or gastric cancer (NCT01795768). FGFR2-amplified GAGA3 (Supplementary Fig. S2A) and GAGA6 PDX tumors were highly sensitive to AZD4547 treatment and showed significant tumor growth inhibition (TGI=100%; Fig. 2A) despite amplification of KRAS and MYC respectively, suggesting that FGFR2 is a dominant driver of tumor growth. In contrast, FGFR2 non-amplified 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 FGFR2 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 terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay by flow cytometry (Fig. 2C) and immunofluorescence (Supplementary Fig. S2B). AZD4547 treatment resulted in a significant increase in TUNEL+ cells in GAGA6 tumors compared to 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 FGFR2 is a dominant driver of tumor growth in spite of MYC and KRAS co-amplification status, and is a druggable target using FGFR 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 favourable response to chemotherapy, most tumours invariably develop drug resistance. Current efforts to understand FGFR inhibitor resistance have focused on in vitro cell line modelling, 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 tumour 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 non-responsive 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 tumour-bearing mice to intermittent AZD4547 treatment over a period of 14 weeks without passaging, after which the tumours developed resistance to AZD4547
and were able to grow in the presence of AZD4547 (Fig. 3D). Drug resistance was achieved at week 10, when tumours continued to grow despite AZD4547 treatment. These tumours 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 tumours was confirmed by FISH (Fig. 3E), demonstrating the emergence of acquired resistance rather than outgrowth of cancer subclones that did not harbour FGFR2 amplification. GAGA6-R PDX tumours 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 signalling 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, 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 tumour survival, we performed siRNA-mediated knockdown of FGFR2 in GAGA6 and GAGA6-R ex vivo tumour cells (Fig. 4A upper panel). 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 lower panel) suggesting the emergence of FGFR2 independence. To determine if FGFR2 had been rendered unresponsive to inhibition by AZD4547, GAGA6-R tumours were treated in vivo with AZD4547 and assayed for activation of FGFR2 as well as downstream signalling 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 AKT and ERK phosphorylation. We also did not observe upregulation or activation by phosphorylation of HER2 and EGFR in GAGA6-R PDX tumours, whether in the presence or absence of FGFR inhibition (Supplementary Fig. S3A). In contrast to parental GAGA6 tumour cells where the phosphorylation of GSK3β, a downstream target downstream 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 tyrosine kinase inhibitor that exerts anti-tumour activity through dephosphorylation of AKT (31, 32), did not lead to dephosphorylation of GSK3β and subsequent cell death (Supplementary Fig. S3B & 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 multi-functional 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 tumour growth in different tumour models (33). Our results suggest that an inactive GSK3β that is phosphorylated at serine 9 is important for tumour 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. Signalling 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 10μM (Supplementary Fig. S3D). However, GAGA6 cells treated with a combination of 1-AKP (at 100nM) and AZD4547 exhibited increased resistance to AZD4547 compared to 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 mediates resistance to AZD4547 treatment and provides 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 protein kinase C (PKC) (34). To investigate the involvement of PKC pathway in GSK3β signalling 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 concomitant downregulation of pro-survival proteins MCL-1 and BCL-XL (Figure 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 to 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 to 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 combination index 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 combination index 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 multi-kinase inhibitor for leukemia treatment with high potency against PKCα/β/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 4l; 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 diffuse-type gastric cancer, 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 diffuse-type gastric cancer models.

In contrast to cell line based studies (29), GAGA6 PDX-tumours 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 modelling 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 non-amplified cells. Activation of compensatory downstream signalling 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 as well as GAGA6-R tumours and were not significantly upregulated or phosphorylated in response to AZD4547 (Supplementary Fig. 3A).

Multi-target 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 tumour 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 signalling. We found that in GAGA6-R cells, inhibition of PKC led to dephosphorylation of GSK3β as well as downregulation of pro-survival proteins MCL-1 and BCL-XL, resulting in increased cell death (Fig. 4F & 4G). 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 signalling is suppressed in GAGA6-R cells, the PKC pathway functions as an alternate signalling pathway to maintain phosphorylation and inhibition
of GSK3β to confer a survival advantage through its downstream pro-survival targets. Rewiring of signalling 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 signalling 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 non-overlapping drug resistance profiles, for instance a multi-kinase inhibitor such as midostaurin in combination with a specific FGFR inhibitor such as AZD4547.

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REFERENCES


Figure 1. **FGFR2 amplification and expression in primary DGC and patient-derived xenograft models of DGC.**

(A) Cytospin preparations of cells before and after CD45 depletion stained with EpCAM (red), and CD45 (green) antibodies, DAPI nuclear counterstain (blue), and analyzed by fluorescent microscopy. Scale bars denote 50µm. (B) Flow cytometry of ascitic cells before and after CD45 depletion, stained with EpCAM, CD45 and isotype control antibodies. (C) Estimated copy numbers of amplified genes in GAGA samples from exome sequencing data. (D) FISH analysis of FGFR2 gene copy number in GAGA1 and GAGA6 PDX models of diffuse gastric cancer. Amplification of FGFR2 was defined as the ratio of FGFR2 (red) signals to CEN10 (green) signals with a ratio ≥2.0. Scale bars represent 10µm. (E) qRT-PCR of FGFR2 expression in DGC PDX tumors of GAGA1, GAGA3, GAGA6 and intestinal type gastric cancer xenograft tumors. Signals are normalized to 18S rRNA internal controls. Error bars represent the mean ± SEM. (F) Immunoblot analysis of FGFR2 protein expression in GAGA1, GAGA3 and GAGA6 PDX tumors with β-actin as loading control.

Figure 2. **Inhibition of FGFR2 signaling by selective inhibitor AZD4547 in FGFR2-amplified diffuse gastric cancer PDX tumors leads to tumor regression.**

Effects of (A) AZD4547 (12.5 mg/kg thrice/week) and (B) cisplatin (2.3 mg/kg once/week) treatment on tumor growth in GAGA1 and GAGA6 PDXs. Statistical analysis was conducted using 2-way ANOVA, where n=10 for drug or control-treated groups. (C) Quantitative determination of TUNEL+ apoptotic cells in AZD4547-treated GAGA tumors. Proportion of TUNEL+ cells as determined by flow cytometric analysis of dissociated AZD4547-treated (AZD) or vehicle-treated (Veh.) xenograft tumors. (D) Immunoblot analysis of FGFR2, FGFR phosphorylation and downstream signalling proteins in GAGA6 PDX tumors treated with AZD4547. Tumors were harvested at indicated time points.

Figure 3. **AZD4547 resistance in cell culture derived (GAGA6-R+) and PDX (GAGA6-R) models**

(A) Establishment of *ex vivo* AZD4547-resistant model GAGA6-R+. GAGA6 tumor cells were cultured *ex vivo* in the presence of AZD4547 (12.5mg/kg thrice/week) until emergence of resistant cells as confirmed by Annexin V apoptosis assay. (B) Chromatograph showing gatekeeper residue p.V564 in GAGA6-R+, GAGA6 and GAGA6-R tumor cells. (C) Immunoblot analysis of cell lysates from parental GAGA6 and GAGA6-R+ cells after *in vitro* treatments with the indicated drugs. Phosphorylation of
FGFR indicates activation of the protein. β-actin serves as a loading control. (D) Establishment of AZD4547-resistant model GAGA6-R. GAGA6 tumor-bearing NSG mice were intermittently treated with AZD4547 (12.5mg/kg) at indicated time points until tumors were able to grow in the presence of AZD4547. Mean tumor volume ± SD (n=3) over time was plotted. (E) FGFR2 copy number in GAGA6 and GAGA6-R PDX models was confirmed by FISH. Scale bars represent 10µm.

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 non-targeting siRNA pool (4 siRNA duplexes each). Cell lysates of the transfected cells were analysed by immunoblot analysis for phospho-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 again 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 (100nM), 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 (100nM) 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. (D) & (E) Statistical analyses were conducted using 2-way ANOVA. (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) Combination index and fractional inhibition values of AZD4547 and H7 drug treatments, computed by CompuSyn software. CI<1, CI=1, and CI>1 indicate synergism, additvity and antagonism, respectively. (I) Effect of midostaurin (100mg/kg daily), AZD4547 (12.5mg/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 2-way ANOVA between two groups.
**A**

Before CD45 depletion

After CD45 depletion

**B**

Isotype controls

Before CD45 depletion

After CD45 depletion

**C**

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**D**

GAGA1 PDX

GAGA3 PDX

GAGA6 PDX

**E**

**F**

FGFR2

β-actin
A

% Live cells (Annexin V+)

AZD4547 concentration

B

ex vivo  in vivo

GAGA6-R+  GAGA6  GAGA6-R

FGF7  F  G  A  G

EGF  E  G

p-FGFR  V  G

FGFR2  G

β-actin

C

ex vivo

GAGA6  GAGA6-R+

BGJ398  -  -  -  +

AZD4547  -  +  +  -  +

FGF7  +  +  +  +  +

p-FGFR  +  +  +  +  +

FGFR2  +

β-actin

D

Tumour volume (mm³)

AZD4547 administration
AZD4547 withdrawal
Tumor passaged

Number of weeks of AZD4547 treatment

E

GAGA6 PDX  GAGA6-R PDX

FGFR2/CEN10/DAP
Figure 4

A. GAGA6 and GAGA6-R expression in cell lines treated with NT FGF2 siRNA or FGFR2 siRNA. The bar chart shows the percentage of Annexin V+ cells for each condition.

B. GAGA6-R expression in vivo with various AZD4547 treatment durations. The table and corresponding Western blot images illustrate changes in p-FGFR, FGFR2, p-AKT, AKT, p-ERK, ERK, p-GSK-3α/β, and GSK-3α/β in response to different concentrations of AZD4547.

C. Schematic diagram showing the interaction between FGF2, AKT, and GSK3α/β.

D. Western blot analysis of p-GSK3α/β and GSK3α/β expression in GAGA6 and GAGA6-R cells treated with 1-AKP or AZD4547.

E. Graph showing the percentage of live cells (Annexin V-) in response to different concentrations of AZD4547.

F. Western blot analysis of various proteins in GAGA6-R cells treated with AZD4547, H7, or both.

G. Graph showing the percentage of apoptosis in GAGA6 and GAGA6-R cells treated with AZD4547, H7, or both.

H. Table showing the fractional combination index for AZD4547 + H7 with different concentrations of GAGA6 and GAGA6-R.

I. Graph showing tumor volume over time after starting drug treatment, comparing vehicle, AZD4547 (12.5mg/kg), Midostaurin (100mg/kg), and AZD4547+Midostaurin (Combi) treatments.
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Acquired resistance to FGFR inhibitor in diffuse-type gastric cancer through an AKT-independent PKC-mediated phosphorylation of GSK3β

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