Acquired On-Target Clinical Resistance Validates FGFR4 as a Driver of Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality worldwide, with no clinically confirmed oncogenic driver. Although preclinical studies implicate the FGF19 receptor, FGFR4, in hepatocarcinogenesis, dependence of human cancer on FGFR4 has not been demonstrated. Fisogatinib (BLU-554) is a potent and selective inhibitor of FGFR4 and demonstrates clinical benefit and tumor regression in HCC patients with aberrant FGF19 expression. Mutations were identified in the gatekeeper and hinge-1 residues in the kinase domain of FGFR4 upon disease progression in 2 patients treated with fisogatinib, which were confirmed to mediate resistance in vitro and in vivo. A gatekeeper-agnostic, pan-FGFR inhibitor decreased HCC xenograft growth in the presence of these mutations, demonstrating continued FGF19-FGFR4 pathway dependence. These results validate FGFR4 as an oncogenic driver and warrant further therapeutic targeting of this kinase in the clinic.

SIGNIFICANCE

Our study is the first to demonstrate on-target FGFR4 kinase domain mutations as a mechanism of acquired clinical resistance to targeted therapy. This further establishes FGF19-FGFR4 pathway activation as an oncogenic driver. These findings support further investigation of fisogatinib in HCC and inform the profile of potential next-generation inhibitors.

INTRODUCTION

Inhibitors of oncogenic driver kinases have significantly improved treatment of many cancers over the past 2 decades. Drugs targeting BCR/ABL, BRAF, EGFR, ALK, and HER2 have dramatically increased therapeutic responses in patients harboring these activated kinases (1). While the activity of targeted compounds across various cancers has aided the elucidation of key oncogenic dependencies, the acquisition of on-target resistance mutations has irrefutably validated disease drivers. From this perspective, resistance to targeted therapies is a double-edged sword that provides biological insights while curbing clinical benefit (1,2).

Identifying and characterizing the mechanisms of acquired resistance is crucial to improving patient care and validating drivers of disease to gain the most value from targeted therapies and improve patient outcomes. The success of next-generation therapies substantiates this approach. Osimertinib has been approved to treat non-small-cell lung cancers that are resistant to gefitinib and erlotinib via a gatekeeper mutation, T790M (3). Avapritinib received Breakthrough Therapy Designation for the treatment of patients with unresectable or metastatic gastrointestinal stromal tumors harboring PDGFRα D842V, which confers resistance to imatinib (4). These examples underscore that despite the presence of resistance, cancers can remain longitudinally addicted to a driver oncogene, and significant benefit can be derived from targeted therapies with unique binding modes that circumvent target resistance mutations.

Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality worldwide (5) with no targeted therapies, as the oncogenic drivers of HCC remain unvalidated. Currently, the multikinase inhibitors sorafenib and lenvatinib are approved in the first-line setting, and nivolumab, regorafenib and cabozantinib have recently been approved as second-line therapies; however, these drugs increase median survival only by a few months, and the identification of primary and acquired resistance mechanisms has been elusive (6-10). Potential drivers of HCC have been identified through genomic profiling, and *FGF19* amplification has been uncovered as an aberrant event in some patients. The FGF19 receptor, FGFR4, and its coreceptor, klotho-β (KLB), are abundantly expressed in normal liver, whereas FGF19 is normally expressed by the ileum upon stimulation by bile acids to repress bile acid production by the liver (11). FGF19 also activates MAPK signaling and induces hepatocyte proliferation crucial for liver repair and regeneration (12). In HCC tumors that overexpress FGF19, constitutive signaling through FGFR4 can lead to unchecked proliferation and neoplastic progression (13,14), and accordingly, antibody- or small molecule-mediated inhibition of FGFR4 reduces tumor growth in liver cancer models (15,16). These findings, coupled with the fact that

FGF19 amplification is associated with a poor prognosis (17), nominate the FGF19 pathway as a driver of disease in HCC and FGFR4 as a potential therapeutic target.

Fisogatinib (BLU-554) is a potent and selective, type I irreversible inhibitor of FGFR4 that has been evaluated in a first-in-human, phase 1 study in patients with advanced HCC (NCT02508467). fisogatinib is well tolerated, and preliminary clinical activity suggested that FGF19 overexpression predicts response to fisogatinib treatment (18), validating FGF19-FGFR4 pathway activation as a driver of disease. Given its kinome selectivity and clinical response rate, fisogatinib is the first targeted investigational agent that can also be used to determine whether on-target mutations in *FGFR4* mediate treatment resistance, further validating the driver status of FGF19-FGFR4 pathway activation. In this study, we describe mutations in the gatekeeper (V550) and hinge-1 (C552) residues of FGFR4 that confer resistance to fisogatinib in the clinical setting, and we validate those mechanisms of resistance in vivo and in vitro. This study further demonstrates continued FGF19-FGFR4 pathway dependency by showing that a gatekeeper-agnostic, pan-FGFR inhibitor can be active against FGFR4 V550M tumors in vivo.

RESULTS

Clinical Identification of Fisogatinib Resistance Mutations in FGFR4

To establish whether acquired FGFR4 mutations contributed to progressive disease (PD) in patients from the fisogatinib phase 1 trial, we sequenced circulating tumor DNA (ctDNA), which is minimally invasive to patients and allows for repeat sample collection. Pre- and post-treatment pairs were available from 31 of 115 total patients enrolled in the study. Baseline characteristics were representative of the total patient population (**Supplementary Table S1**) (18). Twenty-five patients (82%) were positive for FGF19 expression by immunohistochemistry, and 16 patients remained on treatment for at least 6 months. Overall, ctDNA analysis showed evidence of mutations in 12 genes, with 7 mutations being recurrent and elevated at progression (**Supplementary Figure S1**). Seven patients responded to fisogatinib by Response Evaluation Criteria in Solid Tumors (RECIST) criteria, with 6 (27%) partial responses (PR) and 1 (5%) complete response (**Supplementary Table S2**); ctDNA sequencing revealed evidence of acquired on-target resistance in 2 out of 7 cases (29%). The frequency of FGFR4 on-target resistance mutations is comparable to other drivers, such as EGFR (~50-60%) and ALK (~30-50%) in non-small-cell lung cancer (2,3).

Patient 1 was an 81-year-old Caucasian male presenting with Barcelona Clinic Liver Cancer (BCLC) stage C HCC with a nonviral alcoholic cirrhosis etiology. This patient had received 3 prior therapies, including sorafenib and 2 radiotherapy applications after disease progression on sorafenib. This patient's tumor had FGF19 overexpression without *FGF19* amplification (**Fig. 1*A***). Fisogatinib was given at 280 mg/day for the first 8 monthly cycles and 600 mg/day for cycle 9, resulting in a PR with a 44% reduction in tumor size after 16 weeks. At week 32, the patient experienced progressive disease (PD) and the fisogatinib dose was increased to the 600 mg recommended phase 2 dose before discontinuation of treatment. Sequencing of ctDNA at baseline revealed that only wild-type *FGFR4* was detectable before treatment with fisogatinib. By the end of treatment, mutations that corresponded to the gatekeeper (V550M and V550L) and hinge-1 (C552R) residues of FGFR4 were present (**Fig. 1*B*-1*C***).

Patient 2 was a 64-year-old Asian male with BCLC stage C HCC arising from hepatitis B virus infection. This patient's tumor had FGF19 overexpression without *FGF19* amplification at baseline (**Fig. 1*D***). At the time of enrollment, he had received 1 month of sorafenib, which had been discontinued due to toxicity. This patient was given fisogatinib at 600 mg/day for 8 monthly cycles. After 16 weeks, he had stable disease with a 15% radiographic reduction in tumor size. By week 31, the patient experienced PD and was removed from the study. ctDNA sequencing at baseline indicated the presence of wild-type *FGFR4*. Following treatment with fisogatinib, a mutation was detected in *FGFR4* that corresponded with the gatekeeper residue V550M (**Fig. 1*E*-1*F***).

Characterization of Fisogatinib Resistance Mutations

Fisogatinib binds specifically to FGFR4 via a covalent interaction with a unique residue, C552 in the ATP binding pocket (**Supplementary Fig. S2**). We hypothesized that mutations at residue C552 in the FGFR4 ATP binding pocket would reduce fisogatinib binding and inhibitory activity, promoting fisogatinib resistance. A crystal structure of fisogatinib with the kinase domain of FGFR4 combined with enzyme k_{inact} measurements confirmed that the reactive acrylamide moiety of fisogatinib covalently binds with residue C552 within the ATP binding pocket of FGFR4 (19). We hypothesized that mutations at this position would deleteriously erode fisogatinib binding and inhibitory activity. Additionally, given the complementary fit of the dichloro-dimethoxyphenyl headpiece of fisogatinib in a hydrophobic pocket of FGFR4 lined by V550, we predicted that fisogatinib binding would be vulnerable to mutations substituting residue V550 with bulkier amino acids (**Fig. 2*A***).

To corroborate clinical findings and validate that FGFR4 mutations at these key residues arise from persistent exposure to fisogatinib, a resistance screen was conducted in vitro. Ba/F3 cells expressing the fusion protein TEL-FGFR4 were exposed to increasing concentrations of fisogatinib until clonal outgrowth. Ba/F3 clones were considered resistant if the IC_{50} of fisogatinib against a clone was ≥300-fold higher than against the parental line. Fisogatinib is an ideal compound for this screen because it is a highly kinome-selective inhibitor of FGFR4 with a dissociation constant (Kd) of 5 nM that is 134-495-fold higher for FGFR4 than for other FGFRs (**Supplementary Table S3**). Eleven unique mutations were identified across multiple concentrations of fisogatinib, the most frequent occurring in either hinge-1 or gatekeeper residues of FGFR4, as predicted. At the lowest concentration (10 nM), mutations were also identified at residues V548 and A553. The V550L, V550M, and C552R mutations, which were observed in patients treated with fisogatinib, were among the mutants identified in the resistance screen (**Fig. 2*B***). IC_{50} values for the antiproliferative activity of fisogatinib indicated a 300- to 30,000-fold reduction in the potency of fisogatinib against FGFR4 mutants compared with wild-type FGFR4 (**Fig. 2*C***). The pan-FGFR inhibitors infigratinib (NVP-BGJ398) and erdafitinib, and the multikinase inhibitors sorafenib and regorafenib were also tested against FGFR4-mutant Ba/F3 cell lines. Gatekeeper mutations significantly reduced the antiproliferative activity of NVP-BGJ398 and erdafitinib, while the weak activity of sorafenib and regorafenib on FGFR4-mediated proliferation was less affected (**Supplementary Table S4**).

To confirm that FGFR4 hinge-1 mutations prevent covalent binding of fisogatinib, we used ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Unbound, wild-type FGFR4 can be detected at a retention time of 7.83 minutes, while FGFR4 bound by fisogatinib can be detected at a retention time of 11.13 minutes. Fisogatinib covalently bound wild-type FGFR4 in Ba/F3 cells but failed to generate an appreciable peak after 11.13 minutes in cells harboring a representative hinge or gatekeeper mutation (**Fig. 2*D***). A peak at 7.83 minutes was observed in Ba/F3 cells treated with vehicle and in all mutant lines treated with fisogatinib (**Supplementary Fig. 3*A***).

UPLC-MS/MS cannot be used to quantitatively assess the impact of FGFR4 gatekeeper mutations on fisogatinib because it measures only covalent binding. Therefore, we measured the impact of fisogatinib on the enzymatic activity of the FGFR4 V550L gatekeeper mutant. While fisogatinib was highly potent against wild-type FGFR4 (IC_{50} = 4 nmol/L), it only weakly inhibited FGFR4 V550L enzymatic activity (IC_{50} = 3,110 nmol/L), suggestive of impaired binding (**Supplementary Table S5**).

Given the inability of fisogatinib to covalently bind hinge-1 mutant FGFR4 or to inhibit FGFR4 V550L enzymatic activity, we next assessed the impact of FGFR4 mutations on the ability of fisogatinib to inhibit downstream signaling of FGFR4, which normally leads to ERK phosphorylation (20). Fisogatinib markedly decreased ERK phosphorylation in Ba/F3 cells expressing wild-type FGFR4. However, fisogatinib did not affect ERK phosphorylation in those expressing FGFR4 mutations, which is consistent with the observed loss of potency against hinge-1 and gatekeeper mutants (**Fig. 2*E***).

To further elucidate the mechanism of the acquired mutations identified in FGFR4, we characterized fisogatinib resistance in vivo using human HCC Hep3B cells, which have amplified *FGF19* and express FGFR4 and KLB, making them highly sensitive to fisogatinib (**Supplementary Table S6**). Mice bearing Hep3B xenograft tumors were treated with 100 mg/kg fisogatinib orally twice daily until tumors reached approximately 200 mm^3. Fisogatinib administration was then halted until tumors regrew to approximately 700 mm^3. This cycle was repeated 3 times, and then fisogatinib was halted for tumors to grow until day 100 or until they reached 1000 mm^3 (**Fig. 2*F***). Eight mice harbored tumors that grew in the presence of fisogatinib. Sequencing of isolated tumor DNA revealed that each had a missense mutation in FGFR4, with 7 having gatekeeper mutations and 1 having a hinge-1 mutation (**Supplementary Table S7**). Of the gatekeeper mutations observed, only V550L and V550M, which were also observed clinically, were present.

To rule out the possibility that resistance was mediated by an off-target mutation, the clinically relevant FGFR4 V550M mutation was knocked into Hep3B cells using CRISPR-Cas9 gene editing. The resulting knock-in cell line and a wild-type control (**Supplementary Figure 3*B***) were treated with increasing concentrations of fisogatinib. Fisogatinib potently inhibited FGFR4 wild-type but not FGFR4 V550M-mediated signal transduction (**Fig. 2*G***). These findings confirm that FGFR4 gatekeeper mutations are a mechanism of acquired resistance to fisogatinib.

Fisogatinib Resistant Models Retain FGF19-FGFR4 Pathway Dependency

Because FGFR4 gatekeeper mutations were identified as clinically relevant mechanisms of fisogatinib resistance, we hypothesized that an FGFR4 inhibitor that does not bind to the gatekeeper pocket could overcome acquired resistance to fisogatinib and illustrate whether fisogatinib-resistant disease retains FGF19-FGFR4 pathway dependency. LY2874455, a clinical

stage inhibitor with pan-FGFR activity, was assessed as a proof-of-concept tool to examine this principle.

A crystal structure of LY2874455 bound to the kinase domain of FGFR4 revealed that this inhibitor did not bind to the gatekeeper pocket of FGFRs and might not be affected by FGFR4 gatekeeper mutations (**Fig. 3*A***). LY2874455 was similarly potent against wild-type FGFR4 and FGFR4 V550L enzymatic activity, with IC_{50}s of 1.0 nmol/L and 0.4 nmol/L, respectively (**Supplementary Table S8**). To investigate how this enzymatic inhibition translates to an antiproliferative effect in vitro, fisogatinib-resistant Ba/F3 cells were treated with LY2874455 (**Supplementary Table S9**). The antiproliferative activity of LY2874455 was equivalent to that of fisogatinib in cells expressing wild-type FGFR4 but was approximately 25- to 100-fold more potent in cells expressing gatekeeper mutants as published previously (21) and was 700- to 5500-fold more potent against cells expressing hinge-1 mutants compared with fisogatinib (**Fig. 3*B***). LY2874455 was also efficacious against Hep3B tumorspheres generated from CRISPR-Cas9 gene-edited Hep3B cells expressing FGFR4 V550M whereas fisogatinib was not (**Supplementary Fig. S4**). Signal transduction downstream of wild-type and mutant FGFR4 was also similarly affected by LY2874455**,** and this finding was mirrored with wild-type and V550L FGFR4 knock-in Hep3B cells (**Fig. 3*C*** and **3*D,* Supplementary Fig. S5**).

The ability of LY2874455 to function against mutant FGFR4 was further confirmed in vivo. The pharmacokinetic properties and bioavailability profile of LY2874455 make it suitable for in vivo oral dosing. Hep3B cells from the fisogatinib-resistant mouse model harboring a V550M mutation or naïve Hep3B cells expressing wild-type FGFR4 were implanted into mice, which were treated with either LY2874455 (3 mg/kg once or twice daily) or fisogatinib (100 mg/kg twice daily). While V550M-harboring tumors were resistant to fisogatinib, their growth was significantly delayed following treatment with LY2874455. Wild-type FGFR4 Hep3B tumors were sensitive to both compounds (**Fig. 3*E***). These findings demonstrate that a gatekeeper-agnostic inhibitor, such as LY2874455, can directly overcome resistance to fisogatinib in vitro and in vivo and that fisogatinib-resistant disease retains FGF19-FGFR4 pathway dependency.

DISCUSSION

Resistance to therapies targeting oncogenic drivers remains a significant clinical challenge (2). Though this resistance can occur via multiple mechanisms, the result is inevitably reactivation of signaling through pathways that facilitate cellular proliferation and survival. Given that most

kinase inhibitors are small hydrophobic compounds that can rapidly outcompete ATP for their intracellular target, a common form of resistance has been on-target mutation within the kinase domain active site (22). This form of resistance limits the duration of response for patients; however, it also highlights the oncogene dependency of a given cancer and can be used to validate oncogenic drivers. Selective targeted therapies do not currently exist for HCC, and accordingly, clinical validation of potential drivers of this disease has not yet occurred.

Here we uncovered acquired resistance mechanisms with a potent and selective inhibitor of FGFR4, fisogatinib. Fisogatinib was active in patients with HCC who had FGF19 overexpression as measured by immunohistochemistry in a Phase I study (18). Although *FGF19* amplifications are observed in HCC, overexpression is sufficient to induce pathway dependency. The findings of this study validate the FGF19 signaling pathway as an oncogenic driver by identifying FGFR4 kinase domain mutations upon disease progression during fisogatinib treatment. Gatekeeper and hinge-1 mutations were observed in FGFR4 in patients with HCC who initially responded but ultimately progressed on fisogatinib. The identification of 3 unique FGFR4 mutations in 1 patient indicate the possible existence of clonal heterogeneity, demonstrating the utility of liquid biopsies to dynamically assess changes in tumor genetics. These mutations were validated using in vitro and in vivo screening efforts that employed clinically active doses of fisogatinib. Given that gatekeeper mutations were more common than hinge-1 mutations, we speculated that a gatekeeper-agnostic compound with FGFR4 inhibitory activity would effectively illustrate whether a fisogatinib-resistant disease remains dependent on FGF19-FGFR4 pathway activation. We found that LY2874455, which does not bind to the gatekeeper pocket of FGFR4, was active in fisogatinib-resistant in vitro and in vivo models. This observation of differential resistance in the context of consistent oncogene dependence has important implications for treatment sequencing in HCC and supports the clinical development of next-generation FGFR4 inhibitors that maintain FGFR4 selectivity while accounting for resistance mechanisms.

Together, our results corroborate studies implicating gatekeeper mutations as the primary form of resistance for FGFR family members (23-25). In further validating a driver of HCC, we reveal that one of the most prevalent and lethal cancers is amenable to targeted therapy.

METHODS

Fisogatinib Phase 1 study

Clinical data were obtained from a phase 1, first-in-human study (NCT02508467) to define the maximum tolerated dose, recommended phase 2 dose, safety profile, pharmacokinetics, and preliminary antitumor activity of fisogatinib in patients with advanced HCC (18). The study was reviewed and approved by the institutional review board of each clinical site, and written informed consent was obtained from all patients. Eligible patients were ≥18 years of age and had advanced, unresectable HCC with an Eastern Cooperative Oncology Group performance status of 0-1 and available FGF19 status by immunohistochemistry. Patients were accepted regardless of prior sorafenib or regorafenib treatment. Fisogatinib was administered orally, once daily, in 28-day cycles. Dose escalation followed a 3+3 design with doses escalating from140 mg-900 mg. Maximum tolerated dose was established at 600 mg. Radiographic response by computed tomography was evaluated using RECIST version 1.1. Adverse events were graded per Common Terminology Criteria for Adverse Events. ctDNA sequencing was performed using a customized Personal Genome Diagnostics CancerSELECT-60 platform at baseline and was additionally assessed at progression in patients with a possibility of developing resistance due to positive treatment outcome. **Supplementary Table S10** shows the genes that were sequenced in ctDNA samples. Custom additions of panel target regions included FGFR4 alterations. Immunohistochemistry and FISH were assessed by Roche Tissue Diagnostics (Tucson, Arizona) and Q2 Solutions (Morrisville, North Carolina), respectively.

Reagents, Cell Lines, and Cell Culture

Synthesis of fisogatinib was described in the patent application WO2015/061572A1. NVP-BGJ398, erdafitinib, sorafenib, and regorafenib were obtained from MedChemExpress (Monmouth Junction, New Jersey) and LY2874455 from Sigma-Aldrich (St. Louis, Missouri). Cells were obtained from ATCC (Hep3B, PLC/PRF/5, SK-HEP-1, SNU-387, COLO201, and NCI-H2122; Manassas, Virginia), the Japanese Collection of Research Bioresources Cell Bank (JHH-7; Tokyo, Japan), RIKEN Bioresource Center Bank (HUH-7) (Tsukuba, Japan), Sigma-Aldrich (A2780; St. Louis, Missouri), and the Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures (Ba/F3; Braunschweig, Germany). Expression levels of FGFR4, FGF19, and KLB in cell lines were obtained from the Genotype-Tissue Expression (GTEx) Portal (https://www.gtexportal.org/home/). Cells were grown in the media recommended by the vendor and were authenticated annually using short tandem repeat analysis.

Kinase Selectivity Profiling

Fisogatinib was screened at 3 µmol/L using the KINOME*scan* Assay Platform (DiscoveRx, Fremont, California). Dissociation constants (K_d) for test compound-kinase interactions were calculated by measuring the amount of kinase captured on the solid support as a function of the KINOME*scan* test compound concentration. The S(10) score was calculated as described by Karaman et al (26) and is reflective of the number of kinases bound by fisogatinib over the total number of wild-type kinases.

UPLC-MS/MS

FGFR4 and fisogatinib-bound FGFR4 were extracted from Ba/F3 cell pellets lysed in tissue protein extraction reagent and centrifuged to remove cell debris. The lysates were denatured, reduced, and alkylated with iodoacetamide. The samples were then treated with chymotrypsin followed by trypsin to generate FGFR4 peptide (VIVEC$^{[CAM]}$AAK) and fisogatinib-bound FGFR4 peptide (VIVEC$^{[fisogatinib]}$AAK). These peptides were simultaneously quantified with a SCIEX TripleTOF 6600 high-resolution accurate mass UPLC-MS/MS system (Framingham, Massachusetts). A corresponding mass-shifted, stable isotope-labeled peptide (VIVEC$^{[CAM]}$A(d2)A(d2)K) was used as an internal standard (IS). Injections were made using a Shimadzu UPLC system (Kyoto, Japan) with a 20-minute running time. Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in 90:10 acetonitrile/water (v/v). The instruments were controlled by Analyst TF 1.7 (SCIEX). The intensities of the peptides and IS were determined by integration of extracted ion peak areas using MultiQuant 3.0 (SCIEX). The peptide:IS peak area ratio was used for comparison across samples. Calibration curves were prepared by plotting the peptide:IS peak area ratio vs concentration (ng/mL). The model for the calibration curves was linear with ($1/x^2$) weighting. Peptide concentrations (ng/mL) measured in the digested samples were corrected for sample workup and converted to actual protein concentrations in Ba/F3 cell pellets (ng/mg total protein).

Fisogatinib in vitro Resistance Screen

Ba/F3 cells were mutagenized using ENU and seeded in 96-well plates. Fisogatinib was applied at concentrations ranging from 10 nmol/L- 3 µmol/L and incubated until the appearance of resistant clones. Genomic DNA was isolated from resistant clones and sequenced to identify mutations (Genewiz, South Plainfield, New Jersey).

Proliferation Studies

Cell lines were seeded in respective growth media and allowed to grow overnight. Each was

treated with fisogatinib, LY2874455, NVP-BGJ398, erdafitinib, sorafenib, or regorafenib at the doses shown for 2 cell doublings. Viability was assessed using CellTiter-Glo (Promega, Madison, Wisconsin).

Immunoblotting and Antibodies

Cell lines were treated with a given compound at the indicated concentrations for 60 minutes. Cells were pelleted and lysed as described previously (16). Protein concentration was determined using a bicinchoninic acid assay. Western blotting was performed using lysates that were normalized to 50 µg per total protein per lane in loading buffer (Thermo Fisher Scientific, Waltham, Massachusetts). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Thermo Fisher Scientific). Primary antibodies (1:1000 anti-phospho-p44/42 MAPK [Thr202/Tyr204], anti-p44/42 MAPK [3A7]; Cell Signaling Technologies, Danvers, Massachusetts) were incubated overnight at 4°C. Membranes were incubated with IRDye secondary antibodies (LI-COR Biosciences, Lincoln, Nebraska) and imaged on an Odyssey Fc (LI-COR Biosciences).

In vivo Assays

Studies were performed at Wuxi AppTec Co, Ltd. All procedures relating to animal handling, care, and treatment were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Wuxi AppTec Co following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care. Female Balb/c nude mice (Shanghai LC Laboratory Animal Co., Ltd.) were inoculated subcutaneously with Hep3B cells or their fisogatinib-resistant derivative (10×10^6) in 0.2 mL of phosphate-buffered saline mixed with BD Matrigel (50:50; BD Biosciences, San Jose, California). Fisogatinib was dissolved in polyethylene glycol 400; 2-hydroxypropyl-*γ*-cyclodextrin was then added to 20% volume. LY2874455 was dissolved in 10% Acacia and dosed as indicated. Tumor volume was measured at least twice weekly using a digital caliper where tumor volume was determined by the following formula: $V=0.5a\times b^2$, where a and b are the long and short diameters of the tumor, respectively.

STUDY OVERSIGHT

This study was conducted and analyzed by Blueprint Medicines in conjunction with the authors. All authors are responsible for the content of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

RD Kim participated in a speakers' bureau for Eli Lilly and Company; had a consultancy or advisory role with Bristol-Myers Squibb, Bayer, Merck, and Taiho Pharmaceutical; and received research funding from Bayer, Janssen, Bristol-Myers Squibb, Eisai, and Blueprint Medicines. MA Hatlen, O Schmidt-Kittler, C-A Sherwin, E Rozsahegyi, N Rubin, M Sheets, JL Kim, C Miduturu, N Bifulco, N Brooijmans, H Shi, T Guzi, A Boral, C Lengauer, M Dorsch, S Miller, BB Wolf, and KP Hoeflich are employed by Blueprint Medicines and may own stock or other royalties.

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FIGURE LEGENDS

Figure 1. Clinical resistance to fisogatinib is driven by the presence of FGFR4 V550 and C552 mutations. **(A)** Radiographic response to 280 mg of fisogatinib treatment at baseline (top) and at week 16 (middle) in Patient 1. FGF19 status is shown by IHC (lower left) and FISH (lower right). **(B)** ctDNA sequencing analysis before and after treatment with 280 mg of fisogatinib in Patient 1. Sequencing read counts are shown for nucleotides of key codons both before (green) and after (red) treatment with fisogatinib. Nucleotide alterations, corresponding amino acid change, and allele frequency in total cell-free DNA are shown for resistance mutations. **(C)** Radiographic image showing disease progression at week 32, which corresponded with the acquisition of FGFR4 mutations. **(D)** Radiographic response to 600 mg of fisogatinib treatment at baseline (top) and at week 16 (middle) in Patient 2. FGF19 status is shown by IHC (lower left) and FISH (lower right). **(E)** ctDNA sequencing analysis before and after treatment with 600 mg of fisogatinib in Patient 2. **(F)** Radiographic image showing disease progression at week 32, which corresponded with the acquisition of FGFR4 mutations.

Figure 2. FGFR4 V550 and C552 mutations confer resistance to fisogatinib. **(A)** Crystal structure modeling predicts that fisogatinib binding to the ATP binding pocket of FGFR4 would be hindered by mutations at V550 (gatekeeper residue) or C552. **(B)** The percentage of colonies harboring each FGFR4 mutation identified in an in vitro resistance screen using the TEL-FGFR4 fusion protein expressed in Ba/F3 cells. **(C)** The ability of fisogatinib to inhibit proliferation of Ba/F3 cell lines expressing wild-type or mutant FGFR4. **(D)** Time of flight traces acquired from the liquid chromatography-mass spectrometry of fisogatinib or treated Ba/F3 cells expressing the TEL-FGFR4 fusion protein or the TEL-FGFR4 V550M fusion protein. **(E)** Western blot analysis of downstream markers of FGFR4 activity in Ba/F3 cells expressing wild-type or mutant FGFR4 treated with fisogatinib. **(F)** Mice implanted with Hep3B xenografts were treated orally with 100 mg/kg fisogatinib daily until tumors shrunk to approximately 200 mm^3. Fisogatinib dosing was then halted until tumors grew to approximately 700 mm^3, at which point treatment was resumed. This treatment cycle was repeated 3 times, and tumors resistant to fisogatinib were identified. **(G)** Markers of FGFR4 activity were measured with increasing concentrations of fisogatinib in Hep3B cells expressing wild-type FGFR4 or FGFR4 V550M generated through CRISPR-Cas9 gene editing.

Figure 3. A gatekeeper agnostic compound overcomes fisogatinib resistance in vitro and in vivo. **(A)** Crystal structure modeling predicts steric clash between fisogatinib (depicted in

orange) and the acquired methionine in FGFR4 V550M (GK residue); however, a gatekeeper agnostic compound such as LY2874455 (depicted in yellow) is predicted to retain affinity for FGFR4 gatekeeper mutant proteins. **(B)** LY2874455 demonstrated an enhanced ability to inhibit the proliferation of Ba/F3 cell lines expressing mutant FGFR4 when compared to fisogatinib (fisogatinib IC_{50}/LY2874455 IC_{50}). **(C)** Western blot analysis of downstream FGFR4 activity in Ba/F3 cells expressing wild-type or mutant FGFR4 treated with increasing concentrations of LY2874455. **(D)** Markers of FGFR4 activity were measured with increasing concentrations of LY2874455 in Hep3B cells expressing wild-type FGFR4 or FGFR4 V550M generated through CRISPR-Cas9 gene editing. **(E)** Activity of fisogatinib and LY2874455 in mice implanted with Hep3B xenografts or a fisogatinib-resistant Hep3B xenograft expressing FGFR4 V550M generated by CRISPR-Cas9 gene editing.

Figure 1

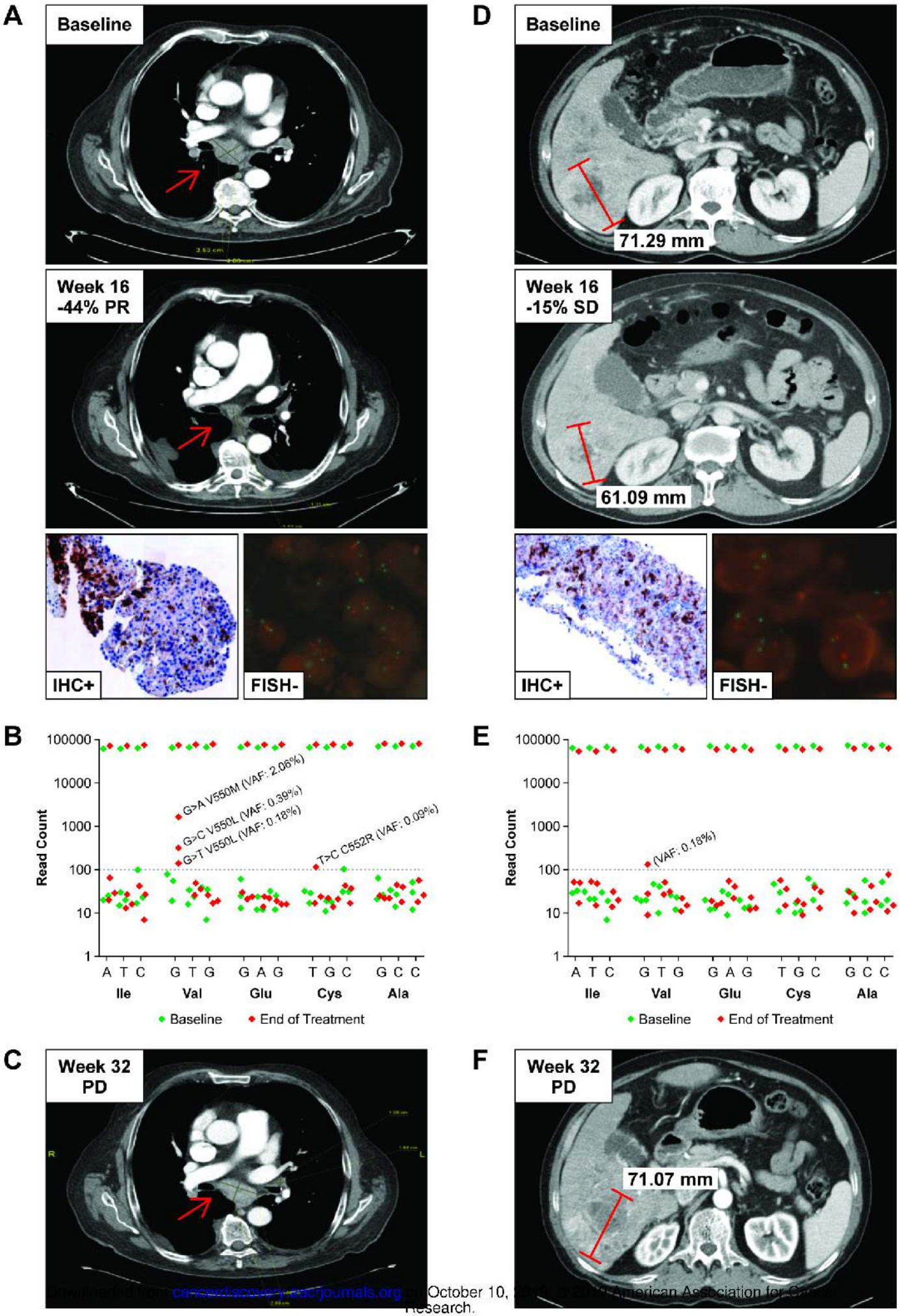


Figure 2

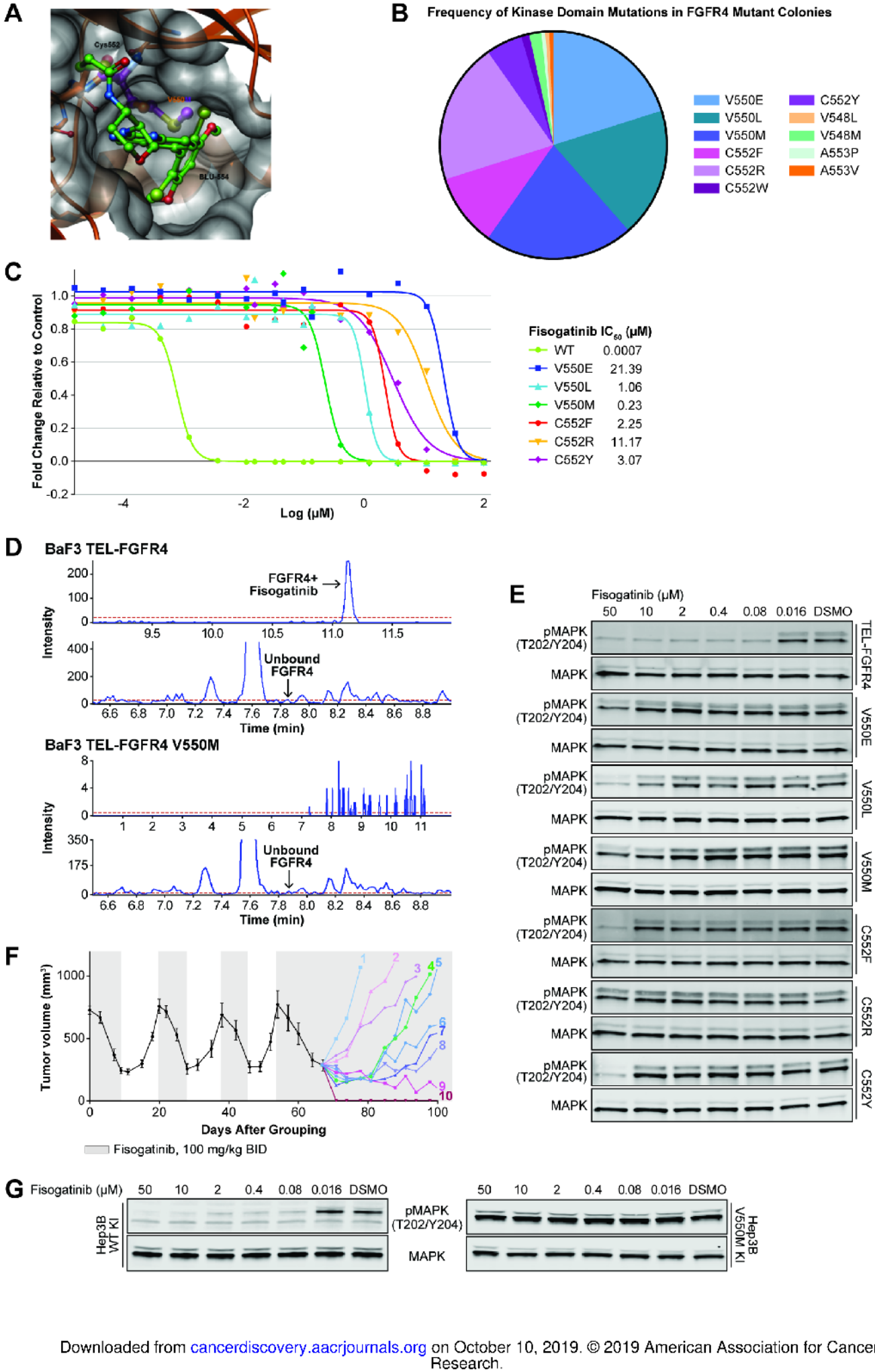
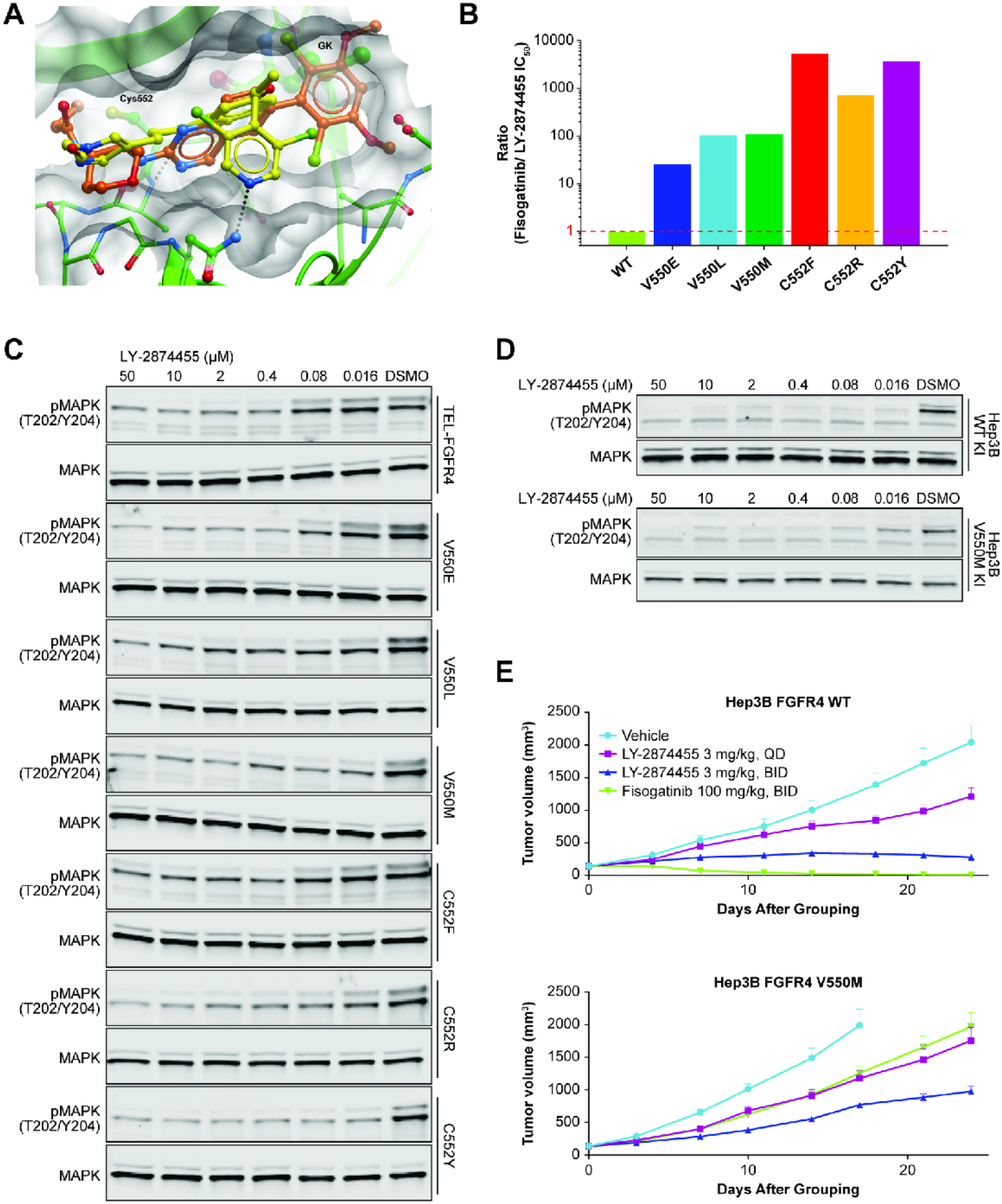


Figure 3



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CANCER DISCOVERY

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