H3B-6527 Is a Potent and Selective Inhibitor of FGFR4 in FGF19-Driven Hepatocellular Carcinoma

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Abstract

Activation of the fibroblast growth factor receptor FGFR4 by FGF19 drives hepatocellular carcinoma (HCC), a disease with few, if any, effective treatment options. While a number of pan-FGFR inhibitors are being clinically evaluated, their application to FGF19-driven HCC may be limited by dose-limiting toxicities mediated by FGFR1–3 receptors. To evade the potential limitations of pan-FGFR inhibitors, we generated H3B-6527, a highly selective covalent FGFR4 inhibitor, through structure-guided drug design. Studies in a panel of 40 HCC cell lines and 30 HCC PDX models showed that FGF19 expression is a predictive biomarker for H3B-6527 response. Moreover, coadministration of the CDK4/6 inhibitor palbociclib in combination with H3B-6527 could effectively trigger tumor regression in a xenograft model of HCC. Overall, our results offer preclinical proof of concept for H3B-6527 as a candidate therapeutic agent for HCC cases that exhibit increased expression of FGF19. Cancer Res; 77(24): 6999–7013. © 2017 AACR.

Introduction

Hepatocellular carcinoma (HCC), which accounts for up to 90% of all primary liver cancers, is a major health issue with an increasing rate of incidence and over 780,000 new cases diagnosed globally each year (1). HCC is a heterogeneous disease with generally poor prognosis and sorafenib, a multikinase inhibitor, is the only approved systemic therapy for advanced HCC patients in the United States (2). Several multikinase inhibitors have been clinically tested in HCC but none have improved activity over sorafenib, and with limited additional treatment options, there is a need for more effective HCC therapies (3). Recent genomic studies of HCC have identified several oncogenic pathways including mutations in the TERT promoter, CTNNB1, TP53, and ARID1A (4). These studies have also identified focal amplification of 11q13.3 chromosomal loci that contains genes including fibroblast growth factor 19 (FGF19) and cyclin D1 (CCND1), suggesting FGF19 as a potential driver oncogene in HCC (5).

FGF19 is a gut-secreted endocrine hormone that acts in the liver through the receptor tyrosine kinase FGFR4 to regulate bile acid synthesis (6). Downstream signaling, upon activation of FGFR4 by FGF19, leads to the inhibition of hepatic enzyme cholesterol 7α-hydroxylase (CYP7A1) expression, resulting in reduced bile acid synthesis (6). This activation of FGFR4 by FGF19 requires the cofactor KLB, which is normally bound to FGFR4 (7). Consistent with the notion that FGF19 is a driver oncogene in HCC, transgenic mice overexpressing FGF19 form liver tumors and genetic ablation of FGFR4 prevents tumor formation (8). In the oncogenic setting, where FGF19 can act in an autocrine/paracrine fashion, MAPK pathway activity and several proliferative genes including EGR1 and c-Fos are known to be regulated by FGF19–FGFR4 signaling (9, 10). These data suggest targeting FGFR4 would have therapeutic benefit in HCC with altered FGF19 signaling. While a number of pan-FGFR inhibitors are being clinically evaluated in a variety of indications, they display poor potency against FGFR4 potentially limiting their application to FGFR1-driven HCC (11). Furthermore, pan-FGFR inhibitors with FGFR4 potency would likely be limited by their off-target FGFR1–3–mediated dose-limiting toxicities (hyperphosphatemia and soft-tissue mineralization), suggesting a need to improve FGFR4 selectivity (11). Emerging studies show that selective inhibition of FGFR4 by antibody and small-molecule approaches are being pursued given the strong link between FGF19, FGFR4, and oncogenesis in HCC (9, 12). Here, we describe the identification of H3B-6527, a highly selective and covalent small-molecule inhibitor of FGFR4 and provide preclinical evidence that HCC with increased FGF19 expression is sensitive to H3B-6527 monotherapy and CDK4/6 inhibitor combination therapy. H3B-6527 is currently undergoing evaluation in a phase I clinical trial in HCC (ClinicalTrials.gov Identifier: NCT02834780).

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

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Materials and Methods

**H3B-6527**

The synthetic procedure for the preparation of H3B-6527 can be found in patent application WO 2015057938 (compound 108, pages 42–45). Detailed structure–activity relationship studies will be described elsewhere.

**CCLE scatter plot**

The log_{2}-transformed mRNA expression of FGFR1, FGFR4, and KLB of 24 CCLE HCC cell lines were purchased from Omicsoft (http://www.omicsoft.com/).

**RNA-seq, cluster, and pathway analysis**

The RNA-seq data was generated by the Illumina HiSeq 4000 RNA Sequencing platform. The fastq files were aligned to both human (GRCh37) and the mouse genomes (GRCm38) using STAR (13) and the aligned BAM files were processed by the in-house xenotool to remove mouse reads and generate the high quality human BAM files. The transcripts abundance was then estimated using Kallisto (14). The RNA-seq data was normalized and log_{2} transformed in R vers 3.2.3 statistical environment (http://www.r-project.org) using edgeR vers 3.16.5 Bioconductor library (15) with trimmed mean of M values (TMM) normalization. The expression profiles of 24-hour time points were used in the gene expression analysis. We excluded nonprotein coding genes and filtered out low expressed genes [genes with log_{2} (rpm) < 2 in all samples] as well as low variation genes (genes with variation < 0.1 across all samples). A total of 5,843 genes were left for the gene expression analysis. For each treatment group, the genes were ranked by fold changes of the treatment comparing with vehicle and the Gene Set Enrichment Analysis preranked was performed using the ranked gene list to obtain the enriched hallmark gene sets (H collection in v5.2 MSigDB) in each group (16, 17). Hallmarks with nominal P < 0.01 in at least one treatment group were reported.

**Kinase assay and competition binding assay**

For kinase assay, the His-TEV-FGFR4 kinase (447–753) was cloned into pFastBac1 (Thermo Fisher Scientific) and expressed in SF9 insect cells. Insect cells were infected with baculovirus at a multiplicity of infection (MOI) of approximately 10 and harvested after 72 hours. Protein was purified by Ni-NTA chromatography followed by size-exclusion chromatography with a sephacryl S-300 column equilibrated in 20 mmol/L Tris pH 8, 20 mmol/L NaCl, and 2 mmol/L TCEP. Peak fractions were pooled and flash frozen in liquid N2. Active FGFR1 kinase was purchased (Thermo Fisher Scientific). IC50 measurements were made using a FRET-based assay using Lance ULight poly-GT as a substrate (PerkinElmer). All measurements were performed at room temperature and run in the presence of 20 pmol/L FGFR1 or 300 pmol/L FGFR4 kinase in a reaction buffer containing 20 mmol/L HEPES pH 7.5, 10 mmol/L MgCl2, 1 mmol/L EGTA, 0.2% Brij35, 0.2 mg/mL BSA, 1 × Roche phosphatase inhibitors, and 2 mmol/L DTT. FGFR1 and FGFR4 were preincubated for 1 hour with inhibitor and the kinase reaction was initiated by addition of poly-GT substrate (100 nmol/L) and ATP (50 μmol/L). Reactions were quenched after 1 hour in Lance detection buffer supplemented with 10 mmol/L EDTA. Substrate conversion was monitored using the LANCE Eu-W1024-anti-phosphotyrosine antibody (2 mmol/L) with an Envision plate reader. Curves were fit using GraphPad Prism software. For competition binding assay, the DiscoverX kinase scan service was utilized (https://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling/kinomescan).

**Mass spectrometry**

For mass spectrometry experiments, 3 μmol/L FGFR4 was incubated overnight at 4°C with 10 pmol/mL H3B-6527 in 50 mmol/L HEPES pH 7.5 and 100 mmol/L NaCl. The protein was desalted and concentrated manually using an OPTI-TRAP column (Optimize Technologies) and eluted in 50% acetonitrile in 0.1% HCOOH. Samples were analyzed by nanocapillary liquid chromatography mass spectrometry on an Easy-nLC 1000 HPLC system coupled to a QExactive mass spectrometer (Thermo Scientific), using an easy spray microflow emitter (Thermo Scientific). The intact protein is eluted at a flow rate of 300 nl per minute with a step gradient starting at 5% and finishing at 50% of buffer (100% acetonitrile in 0.1% formic acid) over 25 minutes. Data was analyzed using BioPharma Finder 1.0 (Thermo Scientific) using a mass tolerance of 0.05 kDa and a cut-off threshold of 15%.

**Crystallography**

The His-TEV-FGFR1-Y563C kinase (458–765) was coexpressed with k-phosphatase in E. coli using a bicistronic construct cloned into pET-28a (EMD Millipore). In this construct, the P-loop mutation C488A and C-lobe mutation C584S were included to improve protein behavior and yields (18). Soluble protein was obtained by overnight induction at 20°C using 0.1 mmol/L IPTG. Cells were harvested and protein was purified using Ni-NTA chromatography followed by overnight TEV cleavage of the His-tag and a polishing subtractive Ni-NTA step to remove the TEV. The flow through was concentrated and injected on a sephacryl S-300 column equilibrated in 20 mmol/L Tris pH 8, 20 mmol/L NaCl, and 2 mmol/L TCEP. Peak fractions were pooled and concentrated to approximately 14 mg/mL and flash frozen in liquid N2. Crystals were obtained at 4°C from 2 + 2 μL hanging drops equilibrated over a reservoir containing 14%–19% PEG 10K, 300 mmol/L (NH4)2SO4, 100 mmol/L MES pH 6.5, and 5% ethylene glycol (19). They grew to full size in 1–3 weeks and were then soaked for 2 weeks in a 10-μL drop containing 9.5 μL of the reservoir solution and 0.5 μL of 30 mmol/mL H3B-6527 formulated in 90% DMSO. The final soaking solution was therefore 1.5 mmol/mL compound with 4.5% DMSO. Crystals were frozen in the soaking solution supplemented with 20% ethylene glycol and data were collected at the Advanced Photon Source, LS-CAT 21-ID-G (Supplementary Table S1). The structure was solved by molecular replacement using MOLREP (20) and refined using Reffmac (21) with ligand coordinates generated using Hi-gand (22). Figure 1D was generated using Chimera (23). Coordinates have been deposited in the Protein Data Bank with accession code 5VND.

**Cell culture and compound treatment**

Hep3B cells (purchased from ATCC), were maintained in Eagle minimum essential medium (ATCC, 30-2003) with 10% FBS. JHH-7 cells (JHH-7 was purchased from Japan Health Sciences Foundation) were maintained in William E Medium (Thermo Fisher Scientific, 12551-032) with 10% FBS. Both cell lines were incubated in a humidified incubator at 37°C with 5% CO2. For the dose response, time course, and wash out experiments, cells were harvested during the logarithmic growth phase, seeded into 6-well
plates (2-mL media/well), and incubated until they reached 80% confluence. Compounds were dissolved in DMSO to yield stock concentrations of 10 mmol/L. DMSO was used as the diluent for all the compound dilutions. The final percentage of DMSO in each well was 0.1% to 0.2%. Wash out was conducted by aspirating the treatment media from the cells, washing the cells with fresh media three times, and then maintaining the cells in fresh media. The 40 HCC cell line panel and 625 cell line panel was conducted using the services of Crown Bioscience (http://www.crownbio.com/oncology/in-vitro-services) and Chempartner (http://www.chempartner.com/biology/cell-biology/cancer-cell-line-panel-screen), respectively. The authenticity of cell lines was confirmed by STR (short tandem repeat) analysis provided by both companies.

**CYP7A1 measurements**

RNA was extracted and purified from 50 to 100 mg tumor fragments using Tri reagent (Ambion) and Omni Bead Ruptor. cDNA synthesis and qRT-PCR was performed using SuperScript Enzyme Mix (Thermo Fisher Scientific) and TaqMan Gene Expression kit. The assay was performed on the Viia7TM Real-Time PCR System (Thermo Fisher Scientific). qRT-PCR data were analyzed using the ΔΔC_{t} method, where each sample was first normalized by subtracting the 18S endogenous control C_{t} value from the CYP7A1 C_{t} value, then subtracting that value from the average vehicle control C_{t} value for that time point. Fold increase was determined by raising 2 to the ΔΔC_{t} value. Results were graphed using GraphPad Prism. Data are expressed as mean ± SEM.

**Western blotting**

To extract protein from the cells for Western blotting, the 6-well plates were placed on ice, the media were aspirated from the wells, and the cells were washed two times with cold 1 × PBS. Cells were directly harvested with sample buffer (2 × NuPAGE LDS sample buffer, 2 × NuPAGE reducing agent, 2 × HALT protease inhibitor, 2 × HALT phosphatase inhibitor, and 2 × EDTA), probe sonicated at 10% amplitude for 10 seconds, and then heated at 70°C for 10 minutes. Samples were loaded and resolved on 4%–15% Criterion TGX precast gels (Bio-Rad). Proteins were transferred onto nitrocellulose membrane using the iBlot 2 Dry Blotting system (Life Technologies). Blots were blocked for 1 hour in LI-COR blocking buffer and probed overnight on a shaker at 4°C with primary antibody (pERK1/2) (Thermo Fisher Scientific, catalog no. 4370) and the antibody was diluted 1:1,000. After washing three times with 1 × PBS-T, they were probed with IRDye 800CW Donkey anti-Rabbit IgG (H+L) secondary (LI-COR), diluted 1:10,000, for 1 hour at room temperature with shaking. Blots were washed again three times with 1 × PBS and then imaged with the Odyssey Infrared Imaging System. The blots were reprobed with p44/42 MAPK (ERK1/2; 3A7) Mouse mAb (Cell Signaling Technology, 4370) and the antibody was diluted 1:1,000. After washing three times with 1 × PBS-T, they were probed with IRDye 800CW Donkey anti-Rabbit IgG (H+L) secondary (LI-COR Biosciences), and imaged again.

**Viability assay and caspase assay**

For both assays, cells were harvested during the logarithmic growth phase and seeded into 96-well plates. Compounds were dissolved in DMSO to yield stock concentrations of 10 mmol/L. DMSO was used as the diluent for all the compound dilutions. The final percentage of DMSO in each well was 0.1%. The viability assay was performed 72 hours after compound treatment by adding 50 μL of CellTiter-Glo Viability Assay reagent (Promega) and caspase assay was performed 24 hours posttreatment by adding 100 μL of Caspase-Glo 3/7 Reagent (Promega) to each well. Plates were shaken for 2 minutes on a microplate shaker and luminescence measured on the Envision plate reader. For viability assay, GI_{50} (the concentration of test article at which cell growth is inhibited by 50% compared with vehicle-treated cells) was calculated according to the publications from NCI (24). Data for caspase assay are expressed relative to DMSO-treated cells.

**IHC**

Five-micron sections of formalin-fixed paraffin-embedded xenograft tissue samples were stained for pERK1/2 (Thermo Fisher Scientific) using a BOND-MAXTM Autostainer (Leica Microsystems) and a Leica ST5020 Multistainer (Leica Microsystems). Briefly, slides were baked at 60°C for 30 minutes, deparaffinized with Bond Dewax solution, and rehydrated. Heat-mediated epitope retrieval was performed in Leica Bond ER1 retrieval at 100°C for 20 minutes. Endogenous peroxidase activity was blocked with 3% 

**FGF19 ELISA**

FGF19 levels were measured in the supernatants (150 μL) using a commercial human FGF19 ELISA kit as directed by the manufacturer (BioVendor). In the event, the signal was beyond the linear range of the assay, the supernatant was diluted, and the final concentration was corrected for the dilution.

**Preparation of H3B-6527, palbociclib, and sorafenib for in vivo experiments**

H3B-6527 was formulated in 0.5% methylcellulose (Sigma, CAS: 9004-67-5) and 0.2% Tween80 (Sigma, catalog no. P4780). Palbociclib was formulated in 25 mmol/L sodium bicarbonate and 15 mmol/L lactic acid solution in distilled water, with 2% Cremaphor. Sorafenib was formulated in equal parts Cremaphor and ethanol as the stock solution. Before use, the stock was diluted in sterile water at 1:3.

**Subcutaneous xenograft generation, dosing, and measurement of antitumor activity**

The BALB/c nu/nu female mice approximately 8-week-old, weighing 18–20 g were obtained from Jackson Laboratory. For the human hepatocellular cancer cell line Hep3B xenografts, Hep3B cells were cultured in RPMI1640 medium containing 10% FBS at 37°C in a 5% CO₂ atmosphere and kept in exponential growth phase. The human hepatocellular cancer cell line JHH-7 was cultured in William E medium with 10% FBS (GIBCO 10099) at 37°C in a 5% CO₂ atmosphere and kept in exponential growth phase.
Figure 1.
Biochemical and structural characterization of H3B-6527. A, Kinome profiling of H3B-6527 at 10 μmol/L with 395 kinases. Red circles, areas of activity as a percentage of control. B, Eleven-point H3B-6527 dose–response curves for kinases identified from the kinome profiling. C, Intact mass spectrometry shows that the untreated protein (top) is 100% modified (bottom) in the presence of H3B-6527. The chemical structure is shown in the inset. D, Crystal structure of H3B-6527 bound in the ATP pocket (PDB 5VND). H-bonds are shown as dashed lines. The refined electron density map (2Fo-Fc) contoured at 1.0σ is continuous between the ligand and the hinge cysteine (C563). The residues (P483-V493) of the p-loop were cropped for clarity.
H3B-6527 for FGFR19-Driven HCC

growth phase. For harvesting, the cells were washed with PBS, incubated with 0.25% trypsin-EDTA, and suspended in a 1:1 mixture of RPMI1640 medium containing 10% FBS and Matrigel (Corning) at a final concentration of 5 × 10⁶ cells/mL for Hep3B and 2.5 × 10⁷ cells/mL for JHH-7. To generate xenografts, 0.1-mL of the inoculum was injected subcutaneously into the right flank region of mice, giving a final concentration of 5 × 10⁶ cells/mouse for Hep3B and 2.5 × 10⁷ cells/mouse for JHH-7. When the mean tumor volume (TV) reached approximately 170 mm³, mice were sorted into treatment groups with 8 animals per group. H3B-6527, palbociclib, or the combination was administered orally either as once-daily or twice-daily schedule as indicated in the figures. The 30 patient-derived xenograft (PDX) models’ one-dose study and the 4 PDX models’ dose–response efficacy study were conducted using 3 animals and 8 animals per group, respectively. For these PDX studies, 6- to 8-week-old female Nu/Nu mice weighing 16–19 g were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd and housed under specific pathogen-free (SPF) conditions. All PDX efficacy studies were conducted using the contract research organization services offered by Shanghai ChemPartner (25). The authenticity of the PDX models was verified using the pathology analysis provided by Chempartner.

Body weights were measured daily and tumor measurements were performed twice weekly. Mice with >20% body weight loss compared with their day 1 body weight or bearing tumors with the longest diameter >2,000 mm were immediately euthanized to prevent any pain or suffering for the animal as according to Institutional Animal Care and Use Committee guidelines defined by the H3 Biomedicine Animal Care and Use Program and study protocol. The tumor volume (TV) in mm³ was calculated according to the following formula: TV = length × width² × 0.5 length: largest diameter of tumor (mm) width: diameter perpendicular to length (mm). The tumor growth inhibition % (TGI) was calculated according to the following formula: Tumor growth inhibition % (TGI) = [(Average control TV day X – Treatment TV day X)/ (Average control TV day X)] × 100, where day X is any day of the treatment.

The antitumor effects of the treatment, partial (PR) and complete regression (CR), stable (SD), and progressive (PD) were defined as follows. PD: 3 consecutive measurements >120% of starting volume or 3 consecutive increasing measurements from best response; SD: 3 consecutive measurements >50% and <120% of starting volume; PR: 3 consecutive measurements <50% of starting volume; CR: 3 consecutive measurements <30 mm³.

Orthotopic xenograft generation, luciferin injection, and imaging

Hep3B cells stably expressing the Luciferease gene (Hep3B-Luc) were inoculated at the left lobe of the liver of female BALB/c nude mice. Hep3B-Luc cells (1.25 × 10⁶) in 25-μL PBS supplemented with 30% Matrigel were used for inoculation. Bioluminescent imaging was performed on the first day of the study and continued on a weekly basis. Treatment with H3B-6527 at various doses given orally twice a day was started 12 days after inoculation when the mean signal intensity was 9.68 × 10¹⁰ photons/second. Luciferin injections were prepared by dissolving 15 mg/mL of luciferin (P-Luciferin, Firefly, potassium salt) in DPBS and sterile filtered through a 0.2-μm filter. Each mouse received 150 mg/kg of luciferin by intraperitoneal injection. After 10 minutes, anesthetized mice were placed in the imaging machine (Xenogen IVIS Lumina XR) and imaged ventrally to detect luminescence signal. Exposure time was 60 seconds. Images were quantified as photons/ second using the Living Image software.

Results

H3B-6527 is a potent, selective covalent FGFR4 inhibitor

The ATP-binding domains of the FGFR family are known to be highly homologous (26). We sought to gain selectivity for the FGFR4 paralog by targeting Cys552, present in the hinge region of the ATP-binding pocket, unique within the FGFR family and found in only 6 other kinases. We evaluated whether an electrophilic small molecule that covalently bound Cys552 would be selective for FGFR4 versus other family members and across the kinome. Using a structure-based design approach focusing on kinase hinge-binding motifs, we discovered templates that present an appropriate vector for attachment of an electrophile to engage Cys552 (27, 28). After iterative rounds of design and synthesis, we chose to focus on an N-aryl-N'-pyrimidin-4-yl urea scaffold, where the introduction of an acrylamide to the ortho-position of the N-aryl substituent provided a compound capable of forming a covalent bond with Cys552 in FGFR4, capturing the residue via hetero-Michael addition of the free thiol. Subsequent optimization of potency and physicochemical properties led to the incorporation of dichlorodimethoxyphenyl and N-ethyl piperazine substituents, ultimately leading to the discovery of H3B-6527.

H3B-6527 was tested at 10 μmol/L against 395 distinct kinases in a competition binding assay that revealed FGFR4 as the top hit (Fig. 1A). Six other kinases, FGFR1-3, TAOK2, JNK2, and CSF1R were also identified with varying binding capacity to H3B-6527 (Fig. 1A). A follow up functional biochemical assay demonstrated robust inhibition of the target kinase FGFR4 with an IC₅₀ value of <1.2 nmol/L and at least 250-fold selectivity over FGFR1-3 (IC₅₀ values of 320, 1,290 and 1,060 nmol/L respectively; Fig. 1B). TAOK2, JNK2, and CSF1R were also less sensitive to H3B-6527 treatment with IC₅₀ values of 690, >10,000, and >10,000 nmol/L, respectively (Fig. 1B). Protein mass spectrometry can provide a direct readout of potential covalent modification of a protein and the stoichiometry of the interaction. When incubated with H3B-6527, FGFR4 underwent 100% monoalkylation, confirming the stoichiometry and covalency of the binding mode (Fig. 1C). The complex was further characterized using a FGFR1 surrogate crystallization system in which the key hinge residue Tyr563 was mutated to cysteine. This simple alteration in FGFR1 effectively mirrors the ATP-binding pocket in FGFR4 and was used to interrogate the binding modes of FGFR4 inhibitors (to be described elsewhere). The complex with H3B-6527 was determined at 2.1 Å resolution (Fig. 1D; Supplementary Table S1). Consistent with mass spectrometry data, the continuous electron density between the thiolate of Cys563 and the acrylamide of H3B-6527 demonstrates the formation of a covalent bond (Fig. 1D). The structure shows how exploitation of this unusual hinge Cys interaction enables H3B-6527 to achieve >250-fold selectivity over FGFR1. This selectivity arises not only from the unique engagement of the cysteine but also from the steric clash that would occur between the acrylamide and the Tyr residue present at the corresponding...
position in FGFR1–3. The compound binds the ATP-binding pocket like other type 1 kinase inhibitors, including the binding mode observed for the covalent FGFR4 inhibitor BLI19931 (9). The N-aryl-N'-pyrimidin-4-yl core forms two hydrogen bonds with the hinge (Fig. 1D), while the dichlorodimethoxyphenyl moiety anchors the compound in the back pocket next to the gatekeeper Val561, Lys514, and Asp641. The solubilizing ethylpiperazinyl group is located at the solvent accessible region as anticipated. Thus, these data provide a structural rationale for the potency and selectivity of H3B-6527.

**H3B-6527 inhibits FGFR4 signaling, proliferation, and leads to apoptosis in a HCC cell line**

As a first step to understand the effect of H3B-6527 on HCC cell lines, we probed the FGFR4 signaling pathway in a HCC cell line, Hep3B. This cell line overexpresses FGFR19 as a result of FGFR19 gene amplification and is a suitable model to study FGFR1–FGFR4 signaling (29). Signaling through FGFR4 leads to phosphorylation and activation of extracellular-signal-regulated kinases (ERK1/2), and also leads to downregulation of CYP7A1 mRNA levels. Thus, both pERK1/2 (Thr202/Tyr204) and CYP7A1 can serve as pharmacodynamic markers for FGFR4 pathway activity (9). First, we tested several concentrations of H3B-6527 in a 24-hour treatment and found a robust increase (approximately 7-fold in comparison with DMSO vehicle) in CYP7A1 transcripts, as measured by qRT-PCR, at 1 nmol/L (Fig. 2A). Higher concentrations of 10, 100 and 1,000 nmol/L led to greater than a 100-fold increase in CYP7A1 transcripts levels, suggesting strong inhibition of the FGFR4 pathway by H3B-6527 (Fig. 2A). Next, we tested the acute effects of 1-hour treatment of H3B-6527 and measured pERK1/2 levels by Western blotting. The levels of pERK1/2 decreased in a dose-dependent manner with maximal inhibition occurring at 100 nmol/L (Fig. 2B). To evaluate the kinetics of FGFR4 inhibition, Hep3B cells were treated with H3B-6527 at 100 and 300 nmol/L for 0.5, 1, 2, 4, 8, and 24 hours and pERK1/2 levels were measured. A rapid decrease in pERK1/2 levels was observed with maximal inhibition at 2 hours at both concentrations and a modest recovery of levels was observed at 8 and 24 hours possibly due to pathway rewiring (Fig. 2C). To evaluate the covalent binding properties of H3B-6527 to FGFR4 in cells, Hep3B cells were treated with 1 μmol/L H3B-6527 for 1 hour and then washed several times with fresh media to remove the compound. The levels of pERK1/2 were measured up to 24 hours post-wash and H3B-6527 showed continued inhibition up to 8 hours consistent with covalent interaction and irreversible inhibition of FGFR4 (Fig. 2D). This was in contrast to the treatment of a noncovalent pan-FGFR inhibitor, PD173074, which led to a 50% reduction in viability of 25 nmol/L (Fig. 2E).

Furthermore, H3B-6527 treatment of Hep3B cells led to robust activation of caspase-3/7, an apoptotic marker, in a concentration-dependent manner, indicating FGFR4 inhibition by H3B-6527 leads to cell death in HCC cell lines (Fig. 2F). In summary, these data show that H3B-6527 inhibits proliferation and leads to apoptosis in a HCC cell line by inhibiting FGFR4 signaling.

**Pharmacokinetics/pharmacodynamics and antitumor activity of H3B-6527 in the Hep3B human HCC xenograft mouse model**

As H3B-6527 has shown FGFR4 pathway modulation in Hep3B cells in vitro, we evaluated the pharmacokinetic and pharmacodynamic properties of H3B-6527 in Hep3B xenografts grown in female nude immunocompromised mice. Three dose levels of H3B-6527 (30, 100, and 300 mg/kg) and a control vehicle group were tested in this study. The study was designed such that five repeat oral doses of H3B-6527 were given on a twice-daily (a dose every 12 hours) schedule and, on day 3, plasma and tumor samples were collected at designated time points and analyzed for H3B-6527 parent compound concentration. H3B-6527 showed dose-proportional plasma exposures and greater than dose-proportional tumor exposures within the dose range evaluated (Fig. 3A). A dose-dependent increase in tumor CYP7A1 mRNA levels was observed and the maximum levels for the 30, 100, and 300 mg/kg dose groups were 46-fold (at 4 hours), 180-fold (at 4 hours), and 509-fold (at 24 hours), respectively, relative to the vehicle levels (Fig. 3A). For the 30 and 100 mg/kg H3B-6527 dose groups, the tumor CYP7A1 levels peaked at 4 hours and then decreased with time. For the 300 mg/kg dose group, tumor CYP7A1 levels remained elevated across each time point (Fig. 3A). Thus, the increase of CYP7A1 levels induced by H3B-6527 was dose-dependent, with higher doses leading to a more sustained response. Inhibition of pERK1/2 (Thr202/Tyr204) by H3B-6527 was also dose dependent, with higher doses leading to more sustained inhibition (Fig. 3B). For the 30 and 100 mg/kg dose groups, the peak inhibition of pERK1/2 was observed at 4 hours and for the 300 mg/kg the peak inhibition was observed already at 1 hour after administration of H3B-6527 (Fig. 3B). Following peak inhibition, the pERK1/2 levels recovered rapidly in the 30 mg/kg group while a slower recovery was observed in the 100 mg/kg group probably due to H3B-6527 clearance (Fig. 3A). No pERK1/2 recovery was seen in the 300 mg/kg group and the levels remained inhibited across each time point likely due to the presence of high levels of H3B-6527 even at later time points (Fig. 3A). In summary, the H3B-6527 pharmacodynamic response as measured by CYP7A1 mRNA and pERK1/2 protein levels was dose dependent, with higher doses leading to sustained responses (Fig. 3A and B).

To test the antitumor properties of H3B-6527, nude mice bearing Hep3B subcutaneous xenografts were treated orally twice daily for 15 days. Treatment with H3B-6527 inhibited Hep3B xenograft growth, with the 300 and 100 mg/kg twice-daily doses significantly inhibiting growth and inducing CR or PR in 8 of 8 and 6 of 8 of mice, respectively (TGI of 115 and 109%, P < 0.05, for the 300 and 100 mg/kg treatment groups, respectively; Fig. 3C). Treatment with 30 mg/kg twice-daily H3B-6527 did not significantly inhibit xenograft growth (TGI of 37%, P > 0.05; Fig. 3C). The vehicle-treated group lost approximately 10% body weight (BW) at day 15 although this BW loss is likely a result of Hep3B tumor-induced cachexia (Fig. 3D; ref. 9). The BW loss induced by Hep3B xenografts was prevented at 300 and 100 mg/kg H3B-6527 where tumor regression was observed on treatment (Fig. 3D). All dose levels of H3B-6527 were well tolerated as no drug-induced BW loss was observed on treatment (Fig. 3D). Next, we tested the antitumor activity of H3B-6527 in an orthotopic liver tumor xenograft model, which is thought to provide a more clinically relevant setting than subcutaneous models (30). Nude mice bearing Hep3B orthotopic xenografts in liver were treated orally on a twice-daily schedule for 28 days and tumor growth was...
measured by the luciferase reporter that has been stably expressed in Hep3B cells. Treatment with H3B-6527 significantly inhibited tumor growth at the 300 and 100 mg/kg twice-daily dose (Supplementary Fig. S1A). Treatment with 30 and 10 mg/kg did not inhibit tumor growth in this study. All dose levels were well tolerated as no drug-induced BW loss was observed (Supplementary Fig. S1B). In summary, these data show that oral treatment of H3B-6527, twice daily, inhibited xenograft growth in a dose-dependent manner in nude mice, with the 300 or 100 mg/kg twice daily, significantly inhibiting tumor growth in both Hep3B

**Figure 2.** H3B-6527 effects in the HCC cell line Hep3B in vitro. A, Relative expression of CYP7A1 24 hours post H3B-6527 treatment at indicated doses. B, pERK1/2 (Thr202/Tyr204) levels 1 hour post H3B-6527 treatment at indicated doses. C, pERK1/2 (Thr202/Tyr204) levels following 100 and 300 nmol/L H3B-6527 at indicated time points. D, pERK1/2 (Thr202/Tyr204) levels at indicated time points following washout of H3B-6527 and PD173074. Initial treatment of both compounds was for 1 hour, denoted by “0 hour.” ERK1/2 levels served as loading control for B–D. E, Representative H3B-6527 dose–response curve for ATP-based cell viability assay. F, Representative H3B-6527 dose–response curve of caspase-3/7–based apoptosis assay.
subcutaneous and orthotopic xenograft model and causing tumor regressions in the subcutaneous xenograft model.

**FGF19 expression is a predictor of H3B-6527 sensitivity**

Having established the sensitivity of the Hep3B HCC model to H3B-6527, we sought to comprehensively study the sensitivity of H3B-6527 by utilizing multiple HCC models. First, 40 HCC cell lines were treated with increasing concentrations of H3B-6527 and growth inhibition was measured by cell viability assay. Any cell line with a GI50 value of \(< 2,000 \text{ nmol/L}\) was considered a sensitive cell line to H3B-6527 and four cell lines met this criterion: Hep3B, SNU-878, Huh-7, and JHH-7 (Supplementary Table S2). CCLE RNA-seq data were available for 24 of the 40 cell lines including the four sensitive lines (31, 32). Examination of these data revealed that the four H3B-6527–sensitive cell lines express high levels of FGF19 mRNA (Supplementary Fig. S2; ref. 9). We evaluated the FGF19 protein levels of these 24 cell lines and found the same four cell lines express the highest levels of FGF19 in culture media (Fig. 4A). We measured the FGF19 protein levels in the remaining 16 HCC cell lines that did not have RNA-seq data and found they failed to produce any detectable levels of FGF19 protein (Fig. 4A). Among these 40 HCC lines, one cell line, Huh-1, produced detectable but much lower FGF19 than the above mentioned 4 cell lines and was not sensitive to H3B-6527 (Fig. 4A; Supplementary Table S2). Consistent with the notion that FGFR4 is the major receptor for FGF19, these 40 HCC cell line panel data indicate FGF19 overexpression can lead to FGFR4 dependence and H3B-6527 sensitivity in vitro.

We next sought to independently confirm these observations using in vivo models. To this end, we utilized 30 HCC PDX models and tested the antitumor activity of H3B-6527 by oral treatment using 500 mg/kg twice-daily dose. Any model where H3B-6527...
FGF19 levels were measured by ELISA and correlated to GI50. Red diamonds, FGF19 overexpression is a strong predictor of H3B-6527 response (Fig. 4B). There were 2 responding models that did not show any FGF19 staining by IHC (Fig. 4B, see Discussion). Most of the nonresponders, 16 of 20, showed no FGF19 staining and only 4 of 20 showed positive FGF19 staining (Fig. 4B, see Discussion). Using the same IHC assay, we then analyzed 231 primary HCC samples to evaluate the frequency of FGF19 positivity in HCC and 44 samples (~20%) scored positive based on similar scoring methods used for the PDX models (Fig. 4C). To strengthen the single-dose PDX models study above, we then performed a dose–response study by choosing 4 PDX models, 3 responding, and one nonresponding model, using four dose levels of H3B-6527. This study showed that the responder models indeed were sensitive to lower doses of H3B-6527 (Fig. 5; Supplementary Fig. S4). In this dose–response study, we included sorafenib, the standard of care in HCC, as a comparator at its MTD in mice and found that H3B-6527 exerts superior efficacy compared with sorafenib (Fig. 5A–D; Supplementary Fig. S5A–S5D; ref. 9). Taken together, the 40 HCC cell line panel and 30 HCC PDX models demonstrate that FGF19 overexpression is a predictive biomarker for H3B-6527 response and evaluation of 231 primary HCC samples suggest approximately 20% of HCC patients may potentially benefit from H3B-6527 treatment.

Having established FGF19 as a strong predictive biomarker for H3B-6527 response in HCC, we searched for additional disease models where the FGF19/FGFR4 axis plays a critical role in viability. To this end, we applied H3B-6527 in a dose–response study to a 625 cell line panel representing 40 cancer types and measured their viability. As before, any cell line with a GI50 value of <2,000 nmol/L was considered sensitive to H3B-6527 and 12 cell lines met this criterion. Validating our earlier observation, 4 HCC cell lines, Hep3B, JHH-7, SNU-878, and Huh-7 showed sensitivity to H3B-6527 (Supplementary Table S3). We identified an additional 8 H3B-6527–sensitive cell lines; SJRH30, RH41, MDA-MB-453, KG1, NALM1, LP1, HEMC SS, and MFE280 (Supplementary Table S3). SJRH30 and RH41 are rhabdomyosarcoma (RMS) cell lines that express high levels FGFR4 due to direct transcriptional activation by the PAX3-FOXO1 oncogenic fusion (RMS) cell lines that express high levels FGFR4 due to direct transcriptional activation by the PAX3-FOXO1 oncogenic fusion (34). The FGFR4 dependence in the KG1 leukemia line, NALM1 lymphoma line, LP1 myeloma line, HEMC bone cancer cell line, and MFE280 endometrial cancer line is a novel and intriguing finding (see Discussion). H3B-6527 did not hit any other line in the 625 cell line panel demonstrating both the selectivity of H3B-6527 and the selective dependence on FGFR4 across cancer types.

**Pallclacib enhances H3B-6527 antitumor activity**

The data described so far indicate that H3B-6527 as a single agent is efficacious in FGF19-overexpressing HCC models. The FGFR4 pathway is thought to restrict proliferation of cells by arresting the G1–S-phase transition through modulation of the cyclin D–CDK4/6 axis, suggesting combined inhibition of FGFR4 and CDK4/6 may provide increased antitumor activity (35). We chose the FGF19-overexpressing JHH-7 cell line xenografts in female nude immunocompromised mice to conduct a combination efficacy study. Hep3B xenografts were not utilized as this cell treatment resulted in tumor stasis or tumor regression is considered a responder and 10 of 30 models fit this criterion with a response rate of approximately 33% (Supplementary Figs. S3 and S4). As our 40 HCC cell line panel viability data suggested that FGF19 overexpression is a strong predictor of H3B-6527 response, we analyzed the HCC PDX models for FGF19 expression using FGF19 IHC. The results of this IHC experiment showed that 8 of 10 responders are positive for FGF19 staining and establishes FGF19 overexpression as a predictive biomarker for H3B-6527 response (Fig. 4B). There were 2 responding models that did not show any FGF19 staining by IHC (Fig. 4B, see Discussion). Most of the nonresponders, 16 of 20, showed no FGF19 staining and only 4 of 20 showed positive FGF19 staining (Fig. 4B, see Discussion). Using the same IHC assay, we then analyzed 231 primary HCC samples to evaluate the frequency of FGF19 positivity in HCC and 44 samples (~20%) scored positive based on similar scoring methods used for the PDX models (Fig. 4C). To strengthen the single-dose PDX models study above, we then performed a dose–response study by choosing 4 PDX models, 3 responding, and one nonresponding model, using four dose levels of H3B-6527. This study showed that the responder models indeed were sensitive to lower doses of H3B-6527 (Fig. 5; Supplementary Fig. S4). In this dose–response study, we included sorafenib, the standard of care in HCC, as a comparator at its MTD in mice and found that H3B-6527 exerts superior efficacy compared with sorafenib (Fig. 5A–D; Supplementary Fig. S5A–S5D; ref. 9). Taken together, the 40 HCC cell line panel and 30 HCC PDX models demonstrate that FGF19 overexpression is a predictive biomarker for H3B-6527 response and evaluation of 231 primary HCC samples suggest approximately 20% of HCC patients may potentially benefit from H3B-6527 treatment.

Having established FGF19 as a strong predictive biomarker for H3B-6527 response in HCC, we searched for additional disease models where the FGF19/FGFR4 axis plays a critical role in viability. To this end, we applied H3B-6527 in a dose–response study to a 625 cell line panel representing 40 cancer types and measured their viability. As before, any cell line with a GI50 value of <2,000 nmol/L was considered sensitive to H3B-6527 and 12 cell lines met this criterion. Validating our earlier observation, 4 HCC cell lines, Hep3B, JHH-7, SNU-878, and Huh-7 showed sensitivity to H3B-6527 (Supplementary Table S3). We identified an additional 8 H3B-6527–sensitive cell lines; SJRH30, RH41, MDA-MB-453, KG1, NALM1, LP1, HEMC SS, and MFE280 (Supplementary Table S3). SJRH30 and RH41 are rhabdomyosarcoma (RMS) cell lines that express high levels FGFR4 due to direct transcriptional activation by the PAX3-FOXO1 oncogenic fusion and were previously shown to be dependent on FGFR4 (33). MDA-MB-453, a breast cancer cell line expresses an activated form of FGFR4, FGFR4-Y367C, which confers dependence on FGFR4 (34). The FGFR4 dependence in the KG1 leukemia line, NALM1 lymphoma line, LP1 myeloma line, HEMC bone cancer cell line, and MFE280 endometrial cancer line is a novel and intriguing finding (see Discussion). H3B-6527 did not hit any other line in the 625 cell line panel demonstrating both the selectivity of H3B-6527 and the selective dependence on FGFR4 across cancer types.

**Palbociclib enhances H3B-6527 antitumor activity**

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line has minimal CDK4/6 activity, likely due to chronic loss of Rb expression and relies on other CDKs for G1–S progression rendering them unsuitable for CDK4/6 inhibitor studies (36). As a first step toward conducting the combination study, we applied H3B-6527 single agent at a high dose of 500 mg/kg twice daily to JHH-7 xenografts and found that oral H3B-6527 treatment led to tumor stasis (Fig. 6A). Next, we reduced the H3B-6527 total daily dose by half and applied 500 mg/kg once daily in combination with palbociclib, an approved CDK4/6 inhibitor, at the MTD dose of 100 mg/kg once daily, both by oral administration. This combination resulted in enhanced tumor growth inhibition as compared with the single-agent arms. Furthermore, the combination led to tumor regressions, whereas the H3B-6527 administered as a single agent twice daily led only to tumor stasis, indicating the combination could increase the best possible response achievable with FGFR4 inhibition (Fig. 6A and B). The combination treatment is well tolerated in mice as no drug-induced body weight loss was observed during the course of the study (Supplementary Fig. S6A and S6B). Examination of the The Cancer Genome Atlas (TCGA) cohort revealed the majority of patients with FGF19-overexpressing HCC expressed appreciable levels of Rb, CDK4, and CDK6, suggesting potential benefit of this combination for the subset (Supplementary Fig. S7).

To probe the mechanism of this enhanced combination effect, we conducted RNA-seq analysis of JHH-7 cell line xenograft tumors following treatment of mice with one dose of either single agents or the combination. Unsupervised clustering of differentially expressed genes showed a distinct pattern in the combination group when compared with the single-agent groups (Fig. 6C). We then performed pathway
Figure 6.
Anti-tumor effects and RNA-seq analysis following H3B-6527 and palbociclib as single agents or in combination in JHH-7 HCC cell line xenografts grown in female nude mice. **A** and **B**, Tumor volume measurements of JHH-7 xenografts at indicated doses and schedule. QD, once daily; BID, twice daily dose; PO, oral dosing. Data represent the mean ± SEM (n = 8). *, P < 0.05 versus vehicle control on the last day of treatment (multiple unpaired t-tests with significance determined using the Holm-Sidak method). **C**, Hierarchical clustering analysis of expressed genes. The samples are ordered by treatment. **D**, Functional annotation using Gene Set Enrichment Analysis. *, functions with nominal P < 0.01.
enrichment analysis of differentially expressed genes, which showed bile acid metabolism as the top upregulated pathway in H3B-6527 single-agent group, a well-known effect specific to the FGFR4 signaling pathway (Fig. 6D) (37). E2F targets scored as the top downregulated pathway; an effect that is common among many receptor tyrosine kinases including FGFR4 (38). Palbociclib single agent also showed significant downregulation of E2F targets as would be expected for a CDK4/6 inhibitor (39). Consistent with the convergence of FGFR4 and CDK4/6 functions on Rb (82–84), the combination groups showed stronger effects on E2F targets as compared with the single-agent groups (Fig. 6D). Moreover, the combination groups showed stronger downregulation of mTORC1 signaling and MYC targets in comparison with the single-agent groups and could explain the increased antitumor activity observed in efficacy experiments (Fig. 6A and B). Taken together, these observations suggest that palbociclib combines well with H3B-6527 to enhance H3B-6527 efficacy in FGFR19-overexpressing HCC models.

Discussion

Patient selection and highly selective drugs are critical factors for the successful clinical development of targeted anticancer agents (3). The remarkable success of biomarker-defined patient enrichment strategies in the treatment of solid tumors with targeted agents has not yet penetrated HCC (3, 40). Sorafenib and regorafenib are the only approved first- and second-line systemic therapies, respectively, in HCC and it is important to note that genomic predictors of their activity are not known, likely due to their multikinase inhibitor mode of action (40, 41). On the basis of their lack of potency against FGFR4, it can be hypothesized that sorafenib and regorafenib will be unsuitable for the treatment of FGFR19-driven HCC (42). Recently, the anti-PD-1 antibody nivolumab has shown durable objective responses with a response rate of approximately 20% and has the potential to become an approved therapy in HCC (43). One criterion that has clearly emerged as a predictor for response to immunotherapy across indications is the mutational load of tumors (44). We examined the mutational load of FGFR19-overexpressing patient samples and found no enrichment of high mutation load, suggesting immunotherapy may be inadequate as a single agent for this HCC subset (45).

In this study, we have shown that inhibiting FGFR4 using a selective small molecule may provide therapeutic benefit to a subset of HCC patients with FGFR19 overexpression. Although pan-FGFR inhibitors are undergoing clinical evaluation for a variety of indications, they are likely unsuitable for FGFR19-driven HCC due to their low potency against FGFR4 (11). We exploited the unique hinge cysteine in the FGFR4 kinase domain to design a covalent inhibitor, H3B-6527, that specifically inhibits FGFR4 and largely spares other FGFRs. Covalent inhibitors directed against a cysteine have recently been developed for other kinases such as Bruton tyrosine kinase (BTK) and EGFR and their success in the clinic has renewed the enthusiasm for covalent inhibitors in cancer therapy (46). Currently, there are three other FGFR4-selective covalent compounds undergoing preclinical and clinical evaluation where the safety and efficacy as a monotherapy will be explored (47–50).

The covalent binding of H3B-6527 to FGFR4 was confirmed directly by mass spectrometry and crystallography. Cellular washout studies supported the covalent mechanism of action and the in vivo xenograft studies showed that H3B-6527 is orally bioavailable and pharmacokinetic/pharmacodynamic correlates with antitumor activity at well-tolerated doses in mice. It is thought that persistent FGFR4 inhibition may lead to excessive production of bile acids resulting in chronic diarrhea, thus limiting the application of FGFR4 inhibitors in the clinic (51). We did not observe any H3B-6527 treatment related diarrhea or body weight loss in mice during the efficacy studies. Factors that could have contributed to this beneficial therapeutic index in mice include shorter half-life of H3B-6527 and differences in the half-life of FGFR4 receptor between tumor and liver. An important element that is thought to complicate HCC therapies is the underlying cirrhosis in the majority of patients. Total bile acid levels are known to be reduced in cirrhosis due to the inhibition of Cyp7A1 in the liver by inflammation (52). Currently, it is not known how FGFR4 inhibition and the potential alterations in bile acid metabolism could impact cirrhosis and affect the outcome of this mode of treatment. Efforts to model this preclinically can be challenging due to the vast differences in bile acid pools and regulation between mice and humans (53).

The selectivity of H3B-6527 to FGFR4 is further illustrated by the 625 cell line panel viability assay where only 12 cell lines showed sensitivity. Among these 12 cell lines, 7 cell lines, belonging to HCC, RMS, and breast cancer lineages have known FGFR19/FGFR4 dependence. Transcriptomic and genomic alterations that lead to FGFR4 dependence (FGF19 overexpression in HCC cell lines and PAX3-FOXO1 fusion in RMS cell lines) are also present in primary samples supporting the notion that H3B-6527 would potentially be useful in these indications. In contrast, the FGFR4-Y367C–activating mutation that confers FGFR4 dependence in the MDA-MB-453 breast cancer cell line is not present in TCGA primary breast cancer samples (34, 54). For the remaining 5 lines representing leukemia, lymphoma, myeloma, bone, and endometrial lineages, dependence on other FGFRs has been described but the existence of FGFR4 dependence was not known (29, 55). We examined the mutational and expression status of FGFR19–FGFR4 pathway components using the publicly available genomic datasets, but did not uncover any meaningful alterations. Future work involving unbiased next-generation sequencing and data mining may uncover the basis for this H3B-6527 sensitivity. Uncovering the basis for this intriguing FGFR4 dependence will potentially broaden the application of H3B-6527 to cancers involving these five lineages.

Initial analysis of 40 HCC cancer cell lines suggested that FGFR1 overexpression is a good predictor for H3B-6527 response. Previous studies have indicated that FGFR4 and KLB expression are also determining factors for FGFR4 inhibitor response (9, 29); however, our analysis using the publicly available RNA-seq data clearly showed that FGFR1 overexpression, as a single biomarker, can predict H3B-6527 response in HCC cell lines. It is noteworthy that FGFR4 and KLB genes are highly expressed in the liver and accordingly the majority of HCC primary samples also display high level expression of FGFR4 and KLB. Although human cancer–derived cell lines are the most widely used models to study the biology of cancer, the clinical relevance of these models has been continuously questioned. Thus, we employed PDX models that are thought to faithfully capture the genotype–response correlation (56). Our study with 30 PDX models again strongly implicated FGFR19 overexpression as a single predictive biomarker for H3B-6527 response. This discovery will help shape and simplify
the patient selection strategies for FGFR4 inhibitor therapy. The mechanism that drives FGF19 overexpression in a limited number of HCC cases (5%) is thought to be the genomic amplification of the 13Q13 locus that contains the FGF19 gene (9, 35, 57). However, other yet-to-be-identified mechanisms must exist as we and others have identified approximately 20% to 30% of HCC overexpress FGF19 (Fig. 4C; ref. 9). Of note, the sensitivity of FGF19 detection assay being applied will determine the frequency of FGF19 overexpression in HCC patients. Although the majority of FGF19-overexpressing PDX models (8 of 12 models) responded to H3B-6527 in our studies, there were some (4 of 12 models) FGF19-overexpressing nonresponders. Genomic analysis did not reveal any alterations in FGFR4, KLB, CCND1, and Rb that could explain this lack of response necessitating further exploration work. While the majority of FGF19-negative PDX models (16 of 18) showed no response to H3B-6527, two models (2 of 18) showed clear response in the absence of any detectable FGF19 staining. A search for activating mutations in FGFR4 or alternative ligands that could activate FGFR4 failed to identify any aberrations and the basis of sensitivity of these 2 models to H3B-6527 remains unknown. Unbiased next-generation sequencing with follow up validation may be needed to discover the underlying biology conferring sensitivity to H3B-6527 in the absence of FGF19 overexpression.

Our work suggests that H3B-6527 can inhibit both cell proliferation and induce apoptosis in HCC cell line models. Although H3B-6527 can induce tumor regression in many PDX models, potentially due to apoptosis, some models showed tumor stasis as the best response. This observation led us to search for a combination partner to improve the response and we tested the CDK4/6 inhibitor, palbociclib, as a potential partner due to its recent success in the breast cancer setting and on the rationale that the majority of growth factor pathways exert their effect through the cyclin D–CDK4/6 axis (58). These experiments led to the discovery that palbociclib can enhance H3B-6527 efficacy and promote tumor regression in JHH-7 model where H3B-6527 as a single agent can only lead to tumor stasis. Gene expression analysis suggested that the combination leads to stronger effects on E2F targets, mTORC1, and MYC pathways that could explain the increased efficacy seen in the combination as compared with the single agents. Palbociclib has been tested in a HCC clinical trial as a single agent but showed only modest activity as a single agent (59). It is noteworthy that palbociclib became a breakthrough therapy in the breast cancer setting as a combination partner to letrozole and in HCC a similar combination approach as described by our studies may be required for significant improvement in clinical efficacy. Our studies using primary HCC samples suggest that a substantial fraction of HCC patients have altered FGF19 expression that could potentially benefit from H3B-6527 monotherapy. Furthermore, the increased activity observed with H3B-6527 in combination with CDK4/6 inhibition provides a rationale for exploration of this combination in early clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E. Corcoran, S. Prajapati, M.-H. Hao, L. Yu, P. Zhu, N. Larsen, J. Wang, M. Warmuth, D.J. Reynolds, P.G. Smith, A. Selvaraj


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.J. Joshi, H. Coffey, E. Corcoran, J. Tsai, K. Ichikawa, M.-H. Hao, S. Bailey, V. Rinkunas, C. Karr, V. Subramanian, W.G. Lai, N. Larsen


Writing, review, and/or revision of the manuscript: J.J. Joshi, H. Coffey, C.-L. Huang, J. Wu, V. Rinkunas, P. Kumar, N. Rioux, A. Kim, W.G. Lai, S. Buonamici, N. Larsen, M. Warmuth, D.J. Reynolds, P.G. Smith, A. Selvaraj

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