Supplementary materials and methods

Antibodies for Western blot, phosphoTyrosine pull-down and FGF19 ELISA

Western blot: anti-FGF19 (Sigma #HPA036082); anti-KLB (R&D Systems #AF5889); anti-FGFR4 (CST #8562); anti-Y436P-FRS2 (Novartis proprietary); anti-Ser473P-Akt (CST #9271); anti- Thr202/Tyr204P-MAPK(CST #9101); anti-MAPK (CST #9102); anti-AKT (CST #9272); anti-β-actin, (Abcam #8227-50); anti-β-tubulin (Sigma #T4026); anti-α-actinin, clone AT6/172 #05-384 Millipore.

PhosphoTyrosine pull-down: anti-phosphoTyrosine clone 4G10 (Millipore #05-321); dynabeads Protein G (Invitrogen #10003D)

FGF19 ELISA: FGF-19 DuoSet DY969 ELISA assay from R&D Systems was used following the indications of the manufacturer.

PhosphoTyrosine / total FGFR4 (pY/tFGFR) ELISA

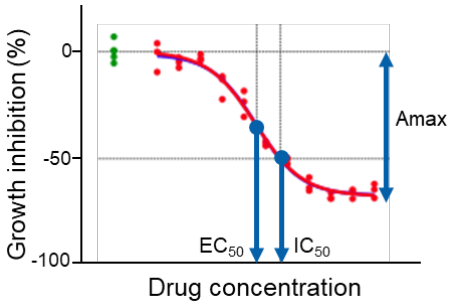
We established a capture ELISA assay to quantify the levels of tyrosine-phosphorylated and total FGFR4 both in cell lines as well as in tumor xenografts. Briefly, separate 96-well ELISA plates (Nunc) were pre-coated with either mouse anti-phospho-tyrosine mAb PY100 (Cell Signaling Technology) or mouse anti-FGFR4 mAb (R&D Systems) at a dilution of 1:100 in PBS without Ca/Mg. Following incubation with 3% Blocker A (MesoScale Discovery) in Tris-buffered saline containing 0.05% Tween-20 (TBST-T), wells were washed with TBST-T. Protein-normalized tissue or cell line samples (200 µg and 100 µg protein for PY100 and FGFR4-capture plates, respectively) were diluted 3:1 with 1% Blocker A in TBST-T (1% MSD blocking buffer) and added to each of the two pre-coated 96-well ELISA plates. After an overnight incubation at 4 °C, wells were washed with TBS-T. ELISA-plates were incubated for 1.5 hours with rabbit anti-FGFR4 mAb (Cell Signaling Technology) diluted 1:1000 in 1% MSD blocking buffer at room temperature. Wells were washed with TBS-T and incubated for 1.5 hours with alkaline phosphatase conjugated goat anti-rabbit IgG F(ab')2 (Invitrogen) diluted 1:20'000 in 1% MSD blocking buffer at room temperature. Wells were washed with TBS-T and deionized H2O, and developed with Tropix CDP-Star Ready to use with Emerald II (Applied Biosystems) for 40 minutes in the dark. Luminescence was recorded on an Infinite M1000 plate reader (TECAN). Wells containing lysis buffer diluted 3:1 in 1% MSD blocking buffer served to determine the assay background. The extent of FGFR4 tyrosine-phosphorylation (pY/tFGFR4) was calculated by dividing the background-corrected signal obtained in the PY100 plate by that obtained in the total FGFR4 plate.

pERK IHC

pErk immunohistochemistry has been performed on the Ventana Discovery XT immunostainer: antigen retrieval was done using CC1 mild conditions, primary antibody (Cell Signaling Technology Cat#4370) incubated at 1/400 dilution for 1 hour at room temperature and detection was done with the OmniMap anti Rabbit HRP/DAB. After counterstaining with hematoxylin/bluing reagent, slides were dehydrated, cleared and mounted with Pertex.

Analysis of cell viability data

For the high throughput cell proliferation assays, all dose-response data was reduced to a fitted model using a propriety decision tree method that is based on the NIH Chemical Genomics Center assay guidelines (20). Fitted models were assessed with a standard Chi^2 test that was also used to determine the best model. All data were manually reviewed as well. Data from the manual cell proliferation assays was analyzed using Xlfit 4 Parameter Logistic Model 205. Parameters derived from the models include EC_{50}, the inflection point of the curve, which equals the concentration of compound needed to produce 50% of the maximal activity; IC_{50}, the concentration where the fitted curve crosses -50% and equals the concentration of compound needed to produce 50% inhibition of cell viability; and Amax, the maximal activity value reached within a model.



shRNA sequences

Non-targetting-1:
NT-1: CCGGAGAAGAAGAAATCCGTGTGAACTCGAGTTCACACGGATTTCTTCTTCTTTTTTA
Non-targetting-2:
NT-2: CCGGGGATAATGGTGATTGAGATGGCTCGAGCCATCTCAATCACCATTATCCTTTTT
Polo-like kinase 1:
Plk1: CCGGGGTATCAGCTCTGTGATAACACTCGAGTGTTATCACAGAGCTGATACCTTTTTAATT
KLB-1:
K-1: CCGGGTAACTTACAGGAGATATACCCTCGAGGGTATATCTCCTGTAAGTTACTTTTTGGAA
KLB-2:
K-2: CCGGCAGTGATAGATAATATATATTCTCGAGAATATATATTATCTATCACTGTTTTTGGAA
KLB-3:
K-3: CCGGACCTATGTCTGAAGCTAAATTCTCGAGAATTTAGCTTCAGACATAGGTTTTTTGGAA
KLB-4:
K-4: CCGGCATGCTCTATCATACTAAATACTCGAGTATTTAGTATGATAGAGCATGTTTTTGGAA
FGF19-1:
F-1: CCGGCACAGTTTGCTGGAGATCAAGCTCGAGCTTGATCTCCAGCAAACTGTGTTTTTGGAA
FGF19-2:

F-2: CCGGGCGGCAGCTGTACAAGAACAACTCGAGTTGTTCTTGTACAGCTGCCGCTTTTTGGAA
FGF19-3:
F-3: CCGGGTCCCAGCTTTGAGAAGTAACCTCGAGGTTACTTCTCAAAGCTGGGACTTTTTGGAA
FGF19-4:
F-4: CCGGGGACGTGCTTCTACAAGAACACTCGAGTGTTCTTGTAGAAGCACGTCCTTTTTGGAA
FGF19-5:
F-5: CCGGGAAACATCTAGAAGTTGTACACTCGAGTGTACAACTTCTAGATGTTTCTTTTTGGAA
FGF19-6:
F-6: CCGGGTCTGATCATAACATTGTAAGCTCGAGCTTACAATGTTATGATCAGACTTTTTGGAA

In vivo PK/PD/efficacy studies

FGF401 was formulated as suspension in 0.5% Tween-80/0.5% carboxymethylcellulose (free base) or as solution in 6% 1 M HCl/100 mM citrate buffer pH 2.5 (citrate salt) and administered orally. Sorafenib was dissolved in Cremaphore EL/ethanol (1:1) and administered orally. Female mice (8-12 weeks of age), Hsd:Athymic Nude-Foxn1nu, Hsd:RH-Foxn1rnu were acquired from Harlan.

For PK/PD studies, treatment was started after randomizing mice into groups of n=2 for vehicle control and each tested dose when the average tumor size had reached approximately 500 mm^3. Animals from each group were sacrificed 2, 4, 8, 12 and 24 h post treatment. Plasma and tumors were sampled and snap-frozen. Concentrations of FGF401 in plasma was determined using UPLC-MS/MS.

For efficacy studies with cell lines-derived xenografts, treatment was started when the average tumor size had reached approximately 150 mm^3 in mice (n=5-6/group) or 250 mm^3 in rats (n=8/group), respectively. Tumor size was measured twice a week with a caliper. Tumor volume was calculated using the formula (*Length x Width) x π/6* and expressed in mm^3. Data is presented as mean ± SEM. Anti-tumor activity is shown as % T/C or % regression and is calculated with the following formulas:

% T/C (percent treatment/control) = $100\times\Delta T/\Delta C$ if $\Delta T > 0$
% Regression = $100\times\Delta T/T_{initial}$ if $\Delta T < 0$
T = mean tumor volume of the drug-treated group on the last day of the study
ΔT = mean tumor volume of the drug-treated group on the last day of the study – mean tumor volume of the drug-treated group on the first dosing day
$T_{initial}$ = mean tumor volume of the drug-treated group on the first dosing day
C = mean tumor volume of the control group on the last day of the study
ΔC = mean tumor volume of the control group on the last day of the study – mean tumor volume of the control group on the first dosing day

The statistical analysis was performed on delta tumor volume (ΔTVol) by comparing the treatment groups to the vehicle control group at endpoint by Kruskal-Wallis followed by Dunn's post-hoc test, or Student's t test. Body weight and tumor volume were recorded twice per week.

The efficacy studies with PDX models were performed at GeneDesign (Shanghai). For each model, tumor fragments of about 15-30 mm^3 were subcutaneously implanted into the flank of Balb/c nude mice. Treatment was started when the average tumor size had reached approximately 200 mm^3 (n=3-4/group) and tumor growth was measured as above. GeneDesign conducted the mRNA expression profiles at BGI (Beijing Genomics Institute, Beijing, China) using Affymetrix human genome U133 Plus 2.0 arrays. The microarray data was normalized using the RMA (robust multi-array average) algorithm. Data for FGF19, FGFR4 and KLB are depicted in Supplementary Table S4

7α-hydroxy-4-cholesten-3-one (C4) and 7-alpha-hydroxycholesterole analysis

C4 analysis in plasma, liver and tumors, and determination of 7-alpha-hydroxycholesterole in enzymatic assays were carried out on an Agilent 1290 Infinity ultra-high performance liquid chromatography (UPLC) system interfaced to an ABSciex API4000 triple-quadrupole tandem mass spectrometer (LC-MS/MS) operated in positive electrospray ionization mode. Chromatography was conducted on an acquity UPLC BEH C18, 1.7µm, 2.1 x 150 mm column at a flow rate of 0.45 mL/min with eluent A (water + 0.1% formic acid) and eluent B (acetonitrileACN + 0.1% formic acidFA) (0-8 min 30% - 40% B, 8-18 min 40% - 70% B, >18 min 100% B). Multiple reaction monitoring transitions were 401-->383 (C4) and 408-->390 (d7-C4), 385-->367 (7-alpha-hydroxycholesterole). Reference standards C4, d7-C4 (internal standard, IS) were obtained from Toronto Research Chemicals, 7-alpha-hydroxycholesterole from Avanti Polar Lipids. All other chemicals were purchased from Sigma-Aldrich. Calibration standards were prepared by spiking defined concentrations of the analytes to 50 µL of aqueous 20% bovine serum albumin solution (BSA, fatty acid free). Plasma samples, tissue homogenates (1:10 w/w in methanol) and calibration standards were processed by addition of 450 µL acetonitrile for protein precipitation and internal standard. Samples were centrifuged at >14'000 × relative centrifugation force for 15 min at 4º C, the supernatants were transferred into new tubes and evaporated to dryness in a vacuum centrifuge. After reconstitution in 60 µL methanol/water 1:1 (v/v) containing 0.1% formic acid (FA), 5 µL were injected into the LC-MS/MS system for analysis.

CYP7A1 activity in mouse liver microsomes

Mouse liver microsomes were prepared according to a standard protocol. Incubations for the enzymatic activity assay contained 100 µL of microsomes (7 mg protein/mL), 900 µL reaction buffer (100 mM phosphate buffer, pH 7.4, 1 mM EDTA, 50 mM sodium fluoride, 5 mM dithiothreitol, 0.015% CHAPS) and 100 µM cholesterol. The reaction was initiated by addition of 100 µL NADPH solution (10 mM) and incubated for 15 min at 37° C. The reaction product 7-alpha-hydroxycholesterole was extracted by petroleum ether, evaporated to dryness in a vacuum centrifuge and reconstituted in methnaol/water 90/10 (v/v) prior to LC-MS/MS analysis.

CYP7A1 mRNA expression in tumors and mouse liver

RNA was isolated using standard methods. Mouse and human specific Taqman assays were purchased from Applied Biosystems and used for real-time polymerase chain reaction (RT-PCR).