Discovery of Selective, Covalent FGFR4 Inhibitors with Antitumor Activity in Models of Hepatocellular Carcinoma

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ABSTRACT: Hepatocellular carcinoma (HCC) accounts for a majority of primary liver cancer and is one of the most common forms of cancer worldwide. Aberrant signaling of the FGF19-FGFR4 pathway leads to HCC in mice and is hypothesized to be a driver in FGF19 amplified HCC in humans. Multiple small molecule inhibitors have been pursued as targeted therapies for HCC in recent years, including several selective FGFR4 inhibitors that are currently being evaluated in clinical trials. Herein, we report a novel series of highly selective, covalent 2-amino-6,8-dimethyl-pyrido[2,3-*d*]pyrimidin-7(8*H*)-ones that potently and selectively inhibit FGFR4 signaling through covalent modification of Cys552, which was confirmed by X-ray crystallography. Correlative target occupancy and pFGFR4 inhibition were observed in vivo, as well as tumor regression in preclinical models of orthotopic and sorafenib-resistant HCC.

KEYWORDS: Selective FGFR4 inhibition, kinase inhibitor, targeted covalent inhibitor, hepatocellular carcinoma

Hepatocellular carcinoma (HCC) accounts for 85–90% of primary liver cancer and is one of the most common forms of cancer with the highest rate of cancer-related mortality worldwide.¹ Pathogenically, the causes for HCC have been commonly associated with chronic liver stress such as viral infection, metabolic disorders, or alcohol abuse. In 2018, there were 841 000 incidents of liver cancer and 781 000 deaths worldwide.² In 2017, in the United States alone, there were approximately 40 000 cases diagnosed and 29 000 deaths.³ The current standard of care for HCC is the multikinase inhibitor sorafenib, which was approved in 2007 based on a three-month improvement in median survival and time to progression.⁴ A significant number of patients present intrinsic resistance to sorafenib treatment and still many others develop acquired resistance.⁵ In 2017, two second line therapies were approved: multikinase inhibitor regorafenib in Japan and the EU, and anti-PD1 immunotherapy nivolumab in the United States. Notwithstanding these notable successes,

and despite many phase 3 attempts to treat HCC, there remains a significant shortage of therapeutic options.⁶

The fibroblast growth factor receptors (FGFRs) are a family of transmembrane receptor tyrosine kinases comprised of FGFR1, 2, 3, and 4 that interact with 18 different fibroblast growth factor (FGF) ligands. FGF/FGFR signaling pathways play essential roles in embryogenesis, metabolism, and tissue homeostasis. Aberrant FGF/FGFR signaling has been implicated in lung cancer, breast cancer, gastric cancer, and HCC, among others.⁷ FGFR4 is the predominant isoform expressed in human hepatocytes and FGF19 is the primary, albeit not exclusive, ligand for FGFR4. FGFR4 pathway

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Figure 1. Structures of representative pan-FGFR and selective FGFR4 inhibitors.

activation requires FGF19 binding as well as the tissue-resident cofactor β -klotho (KLB). The combination of FGF19/FGFR4/KLB is uniquely effective in driving hepatocyte proliferation, dysplasia, and neoplasia. These effects can be reversed *in vitro* and in animal models via neutralization of FGF19 and/or inhibition of FGFR4, supporting the hypothesis that inhibition of FGFR4 signaling may provide therapeutic benefit in certain cancers.^{8–10} Furthermore, through selective inhibition of FGFR4 over FGFRs 1–3, one could achieve therapeutic benefit without encountering dose limiting toxicities associated with inhibition of FGFR1 and FGFR3, particularly hyperphosphatemia and hypocalcemia. Taken together, these data collectively support the therapeutic rationale for selective inhibition of FGFR4 in patients with FGF19 amplified HCC.

FGFR proteins are highly homologous with sequence similarity of approximately 88% and sequence homology of 68%, on average. When our program was initiated, only pan-FGFR inhibitors were reported and often exhibited weaker potency against FGFR4 compared to FGFRs 1–3. Examples include PD173074, BGJ-398, and AZD4547 (Figure 1). In recent years, the discovery of potent and selective FGFR4 inhibitors has been actively pursued by numerous groups and has been the topic of multiple reviews.¹¹ A diverse set of small molecule inhibitors has been identified (Figure 1), including several that are currently being evaluated in clinical trials (BLU554, H3B-6527, and FGF401). In a recent Phase 1 study, BLU-554 was shown to elicit clinical responses in patients with advanced FGF19 positive HCC.¹² Herein, we report the design, synthesis, and biological evaluation of a novel series of potent and selective 2-amino-6-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one FGFR4 inhibitors that promote tumor regression in sorafenib-resistant and orthotopic human tumor xenograft (HTX) models.

Covalent drugs have proven successful as targeted therapies for a variety of malignancies with known oncogenic drivers, achieving high levels of target engagement and protracted target occupancy *in vivo*. In addition, covalent inhibition may provide an advantage in cases of acquired drug resistance.¹³ FGFR4 contains a unique cysteine (Cys) residue numbered 552. FGFRs 1–3 present a tyrosine (Tyr) at the same position. Furthermore, Cys552 is conserved in just 5 other human protein kinases, including MK2, MK3, S6K2, STK40, and TTK. Thus, covalently targeting the Cys552 in FGFR4 is an appealing strategy for achieving selective inhibition of FGFR4 both with respect to isoform and kinome selectivity.

Through structure-based design, we were able to identify an ATP competitive 2-amino-6, 8-dimethylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one scaffold that achieved covalent modification of Cys552 and inhibition of the catalytic activity of FGFR4. Optimization of cellular potency, selectivity, and pharmacokinetic properties resulted in identification of compound 1 which inhibits pFGFR4 signaling in cells with IC_{50} = 9 nM and >100 fold selectivity over FGFRs 1–3. FGFR2 IC_{50} was used as a surrogate marker of FGFR 1–3 inhibition in cells, as potencies were generally similar.

A cocrystal structure of compound 1 covalently bound to the kinase domain of human FGFR4 was determined by X-ray crystallography at 1.9 Å resolution (Figure 2, PDB 6V9C). The

Figure 2. Co-crystal structure of compound 1 in complex with FGFR4 determined to 1.9 Å resolution. Compound 1 forms a covalent bond to Cys552 and two H-bonds with Ala553 in the hinge region of the ATP binding pocket. The dimethoxyphenyl group binds in a hydrophobic pocket and forms H-bond interactions with Asp630.

structure shows that compound 1 binds to the DFG-in conformation of the FGFR4 kinase domain. The acrylamide forms a covalent bond with Cys552 in the hinge region of the protein. Additionally, the acrylamide forms hydrogen bonding interactions with Ala553 in the hinge region and Arg483 located on the N lobe. The carbonyl of the pyridopyrimidinone core forms a water-mediated hydrogen bond with Asp630 of the DFG region of the activation loop. The dichloro- and dimethoxy-substituted benzene resides in a hydrophobic region formed by Phe631, Met524, and multiple

Table 1. Kinase Inhibitory Activities of Compounds

Compound	R ₁	R ₂	Enzymatic IC ₅₀ (nM) ^a		Cellular IC ₅₀ (nM) ^b		Compound	R ₁	R ₂	Enzymatic IC ₅₀ (nM) ^a		Cellular IC ₅₀ (nM) ^b	
			FGFR4	FGFR2 ^c	FGFR4	FGFR2 ^c				FGFR4	FGFR2 ^c	FGFR4	FGFR2 ^c
1			6.5	505	8.8	842	6			13	2156	29	3602
2		---	2340	394	2926	377	7			588	10000	685	5000
3		---	3161	776	2212	1219	8			10	5132	25	5000
4		---	73	247	35	359	9			4.1	672	11.4	1172
5		---	6	102	20	218	10			6.5	563	7.2	617

^aValues determined using an Omnia Kinase Assay at 250 μ M ATP. ^bpFGFR4 and pFGFR2 formation in Huh7 and KATO cells, respectively. ^cInhibition of FGFR2 phosphorylation was used as a surrogate for FGFR1 and FGFR3, as potencies against these off-targets were similar.

Scheme 1. Synthesis of Compound 1^a

^aReagents and conditions: (a) MeNH₂, DCM, 0 °C, 30 min, 100%; (b) LiAlH₄, THF, 15 min, 53%; (c) MnO₂, DCM, 24 h, 75%; (d) **13**, K₂CO₃, DMF, 110 °C, 4 h, 63%; (e) SO₂Cl₂, DCM, 0 °C, 30 min, 70%; (f) KOH, THF/H₂O (1:1), 0 °C, 4 h, 83%; (g) POCl₃, CH₃CN, 90 °C, 6 h, 95%; (h) **14**, DIPEA, NMP, 90 °C, 2 h, 71%; (i) TFA, DCM, 2 h, 97%; (j) acryloyl chloride, DCM, DIPEA, −78 °C, 10 min, 67%.

hydrophobic residues located on the β -sheet of the N-lobe, including Val481. In addition to hydrophobic interactions, one methoxy group forms a hydrogen bond with the main chain NH of Asp630.

Structural alterations and a matched-pair analysis with compound **1** furthered our understanding of structure–activity relationships (SAR) (Table 1). Covalency was critical for potency as well as selectivity, demonstrated by a comparison of **1** with its reversible propionamide analogue **2**. Positioning of the acrylamide was impactful, and a reversal in the absolute configuration of the adjacent stereocenter, as shown in compound **3**, resulted in a significant loss in potency and selectivity. Covalent modifiers with increased cysteine reactivity such as vinylsulfonamide **5** led to erosion of

selectivity without a gain in potency. The penta-substituted benzene in the hydrophobic back pocket of FGFR4 could be modified to maintain or attenuate potency and selectivity. Removal of either chloro or methoxy groups led to reduced potency. However, replacing either or both chlorines with fluorine resulted in similar levels of potency and selectivity.

The synthesis of compound **1** is shown in Scheme 1. Chloropyrimidine **11** was treated with methylamine to displace the chloride in quantitative yield. The methylamine adduct was then subjected to reduction with LiAlH₄ and oxidation with MnO₂ to furnish aldehyde **12** in 40% overall yield in two steps. Thermal condensation of **12** with **13** in the presence of K₂CO₃ provided **14** in 63% yield. Simultaneous oxidation and chlorination of **14** was achieved with SO₂Cl₂ in 70% yield.

The sulfone moiety in **55** was converted to a chloride in sequential hydrolysis and chlorination steps with 79% overall yield. Coupling of **16** with amine **17** furnished **18** in 71% yield. Boc deprotection followed by acrylamide formation provided compound **1** in 65% yield over two steps.

Pharmacokinetic analysis of **1** revealed a high C_{max} , low clearance, and 20, 12, and 27% oral bioavailability in mouse, rat, and cyno, respectively (Table 2). Pharmacodynamic (PD)

Table 2. Pharmacokinetics of Compound 1 in Mouse, Rat, and Cynomolgus Monkey

species	mouse		rat		cyno	
route	IV	PO	IV	PO	IV	PO
dose (mg/kg)	3	10	3	10	3	10
CL (mL/min/kg)	17.2		9.0		8.4	
V_{ss} (L/kg)	0.63		0.30		1.1	
$T_{1/2}$ (hr)	2.1	1.8	2.1	3.2	4.8	2.0
C_{max} (mg/mL)	6270	423	9130	588	5100	2820
AUC (ng·hr/mL)	2910	2960	5610	2230	6420	5660
%F		20		12		27

effects were studied by measuring target occupancy and inhibition of pFGFR4 signaling in Huh7 tumor-bearing mice after single dose oral administration at 2.5, 10, 30, and 100 mg/kg. The degree of target occupancy, determined by ratio of unbound to total FGFR4, was in close correlation with inhibition of pFGFR4 signaling, measured by Mesoscale Discovery (MSD) assay, with max inhibition observed at C_{max} . Target occupancy >80% was sustained for approximately 10 h after a single dose at 100 mg/kg (Figure 3).

Figure 3. pFGFR4 inhibition and target occupancy in Huh7 tumors after a single oral administration (100 mpk) of **1**.

The antitumor activity of **1** was studied in two human tumor xenograft (HTX) models of HCC, an orthotopic model of Hep3B tumors and a subcutaneous (SC) model of sorafenib resistant Huh7 tumors, in SCID mice. In a Hep3B model, 100 mg/kg of inhibitor **1** administered PO twice daily resulted in tumor regression and sustained growth inhibition over 28 days (Figure 4). Sorafenib-resistant tumors were established by orally administering sorafenib to animals bearing Huh7 tumors, at a dose of 100 mg/kg daily for 14 months until the tumors were completely resistant to sorafenib treatment. After establishment of sorafenib-resistant tumors, oral

Figure 4. Antitumor activity of compound **1** (100 mg/kg BID) in an orthotopic Hep3B HTX model in SCID mice on day 28 of treatment.

administration of **1** at 10, 30, and 100 mg/kg BID for 11 days resulted in dose-dependent growth inhibition of resistant tumors. Tumor regression was observed at 30 and 100 mg/kg, with % $\Delta T/\Delta C = 67\%$ and -70% , respectively ($p < 0.0001$). As expected, treatment with sorafenib at 100 mg/kg once daily did not provide any benefit (Figure 5).

Figure 5. Antitumor activity of compound **1** in an SC sorafenib resistant Huh7 HTX model in SCID mice.

In summary, a series of covalent and selective FGFR4 inhibitors were identified. Potent inhibition of FGFR4 phosphorylation was achieved enzymatically and in cells. Isoform and kinome selectivity were achieved, in part, through covalent modification of the unique Cys552, which was confirmed by X-ray crystallography. In addition, compound **1** achieved sufficient *in vivo* exposure to achieve high levels of FGFR4 occupancy and pFGFR4 inhibition, in tumor bearing mice. In orthotopic and sorafenib-resistant HCC HTX models, oral administration of **1** resulted in notable and sustained tumor regression. Collectively, these data support the potential utility of **1** for treatment of FGF19/FGFR4 driven diseases including sorafenib resistant HCC.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00601>.

Synthetic procedures and analytical data of selected compounds, conditions for all biological assays, and X-ray crystallographic methods and statistics, kinome inhibition profiling, *in vivo* PK/PD protocols, and tumor growth inhibition studies protocols (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; DMPK, drug metabolism and pharmacokinetics; HCC, hepatocellular carcinoma; PK, pharmacokinetics; PD, pharmacodynamics; EU, European Union; anti-PD1, programmed cell death protein 1 antibody; KLB, cofactor β -klotho; DFG, Asp-Phe-Gly motif; Boc, *tert*-butoxycarbonyl protecting group; DIPEA, *N,N*-diisopropylethylamine; NMP, *N*-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; MSD, mesoscale discovery; cyno, cynomolgus monkey; CL, clearance; V_{ss} , volume of distribution; $T_{1/2}$, half-life; C_{max} , maximum plasma concentration; AUC, area under the curve; %F, oral bioavailability; HTX, human tumor xenograft; QD, once a day; SCID, severe combined immunodeficiency.

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