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Investigation of Covalent Warheads in the Design of 2-Aminopyrimidine-based FGFR4 Inhibitors

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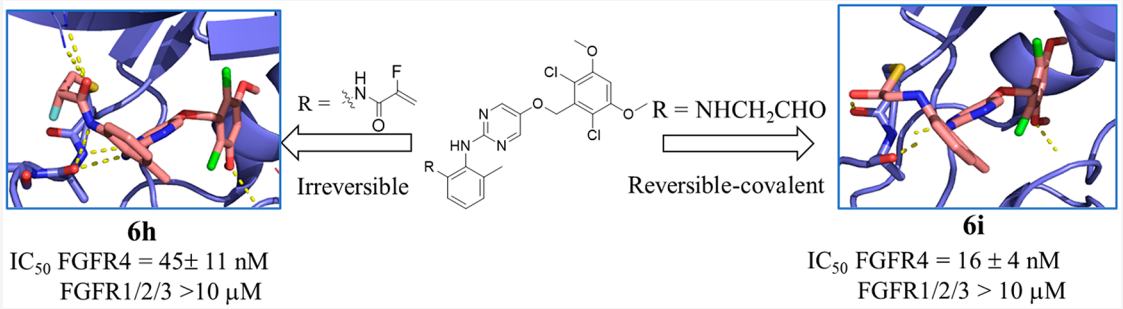

ABSTRACT: Covalent kinase inhibitors are rapidly emerging as a class of therapeutics with clinical benefits. Herein we report a series of selective 2-aminopyrimidine-based fibroblast growth factor receptor 4 (FGFR4) inhibitors exploring different types of cysteine-targeting warheads. The structure−activity relationship study revealed that the chemically tuned warheads α-fluoro acrylamide, vinylsulfonamide, and acetaldehyde amine were suitable as covalent warheads for the design of selective FGFR4 inhibitors. Compounds **6a**, **6h**, and **6i** selectively suppressed FGFR4 enzymatic activity with IC_{50} values of 53 ± 18, 45 ± 11, and 16 ± 4 nM, respectively, while sparing FGFR1/2/3. X-ray crystal structure and MALDI-TOF studies demonstrated that compound **6h** bearing the α-fluoro acrylamide binds to FGFR4 with an irreversible binding mode, whereas compound **6i** with an acetaldehyde amine binds to FGFR4 with a reversible covalent mode. **6h** and **6i** might provide some fundamental structural information for the rational design of new selective FGFR4 inhibitors.

KEYWORDS: *Warheads, FGFR4, fluoro acrylamide, reversible covalent inhibitors*

Protein kinases have become important therapeutic targets for many proliferative human diseases, especially cancer and inflammation.[1] However, designing selective kinase inhibitors is challenging because the majority target the highly conserved ATP-binding pocket.[2] Covalent targeting of a noncatalytic cysteine in a kinase represents a successful strategy in some oncology indications, with reduced risk of toxicity and higher selectivity. Over the past two decades, seven covalent kinase inhibitors, such as EGFR inhibitors (afatinib, neratinib, osimertinib, and dacomitinib) and BTK inhibitors (ibrutinib, acalabrutinib, and zanubrutinib), have been approved by the FDA.[3] Covalent kinase inhibitors typically comprise a scaffold connected to an electrophilic functional group, also known as a "warhead", such as an acrylamide moiety and its derivatives.[4] These form a covalent bond with reactive residues in the kinase active site including cysteine, lysine, and others.[5] However, covalent inhibitors can cause serious toxicity due to their nonspecific conjugation to proteins and cellular macromolecules.[6] To reduce the risk of toxicity and improve their selectivity, the rational design of selective covalent kinase inhibitors is critically important.

The fibroblast growth factor receptor (FGFR) family comprises four members, FGFR1, FGFR2, FGFR3, and FGFR4, that are high affinity receptors for the fibroblast growth factors (FGFs), which are involved in many fundamental biological processes.[7] Among these isoforms, FGFR4 specifically utilizes FGF19 as the intracellular ligand to stimulate and activate the signaling pathway. Previous studies have demonstrated that FGFR4 and FGF19 signaling aberrations are a valid oncogenic driver of cancer, especially for hepatocellular carcinoma (HCC).[8] Therefore, FGFR4 has

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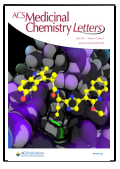

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become a novel potential target for the treatment of HCC. As a result, selective FGFR4 inhibitors are of significant interest as an alternate therapeutic strategy for the development of pan-FGFR inhibitors, seeking to avoid the side effects associated with FGFR1/2/3 on-target inhibition.

Although FGFR1/2/3/4 possess high sequence homology, one poorly conserved cysteine located at position 552 of FGFR4 (tyrosine in FGFR1/2/3) has been demonstrated to be an effective site to obtain FGFR4 isoform selectivity.[9] Recently, several selective FGFR4 inhibitors have been reported that form a covalent bond with Cys552 in FGFR4,[10] such as BLU9931 (**1a**)[9] and BLU554 (**1b**),[11] H3B-6527 (**2**),[12] aminopyrimidine derivative (**3**),[13] developed by our group, and aldehyde FGF401 (**4**) (Figure 1).[14] FGF401

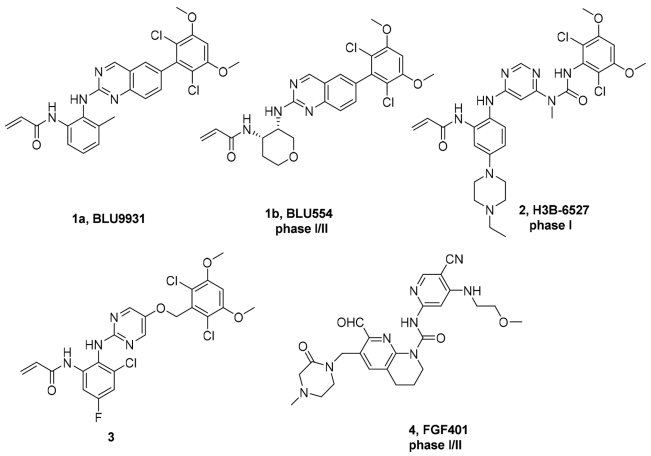


Figure 1. Structures of representative selective covalent FGFR4 inhibitors.

is a selective reversible covalent FGFR4 inhibitor designed to overcome the rapid FGFR4 protein resynthesis rate observed in some HCC cell lines.[15] An inhibitor with a rational toxicity profile that allows for frequent dosing to achieve complete and continuous FGFR4 inhibition to reach maximum antitumor efficacy is urgently required. Despite the successful design of targeted covalent FGFR4 inhibitors, most are still in early clinical trials. No FGFR4 selective inhibitors have been approved by the FDA to date. To explore the development of FGFR4-selective inhibitors with improved isoform binding specificity, we investigated the cysteine binding efficiency of different warheads in FGFR4. Several irreversible and reversible covalent warheads targeting cysteine, such as α,β-unsaturated acrylamide and its derivatives among others, have been developed and recently reviewed[16] for their promise in the design of covalent and reversible covalent inhibitors. As described herein, we designed and synthesized a series of molecules based on the 2-aminopyrimidine scaffold seeking to incorporate different types of potential warheads instead of the original acrylamide warhead (Figure 2).

The synthesis of the designed compounds **6a−6j** is outlined in Scheme 1. In brief, the commercially available 2-chloropyrimidin-5-ol (**8**) was reacted with 3-(bromomethyl)-2,4-dichloro-1,5-dimethoxybenzene (**7**), giving the corresponding 2-chloro-5-((2,6-dichloro-3,5-dimethoxybenzyl)-oxy)pyrimidine (**9**) through a nucleophilic substitution reaction.[11] Intermediate **9** was then coupled to 2-methyl-6-nitroaniline (**10**) to afford intermediate **11** through a Buchwald−Hartwig coupling. The iron dust reduction of **11**

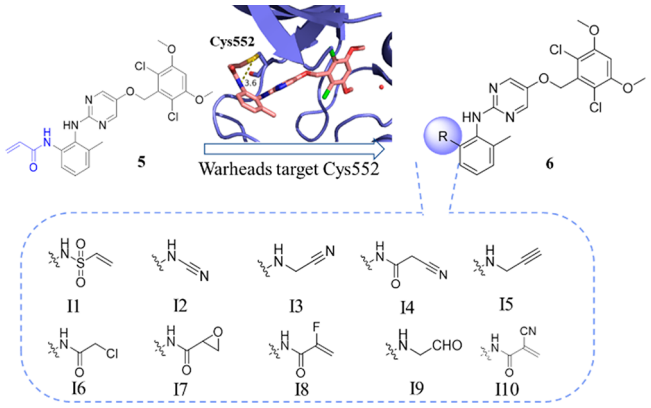
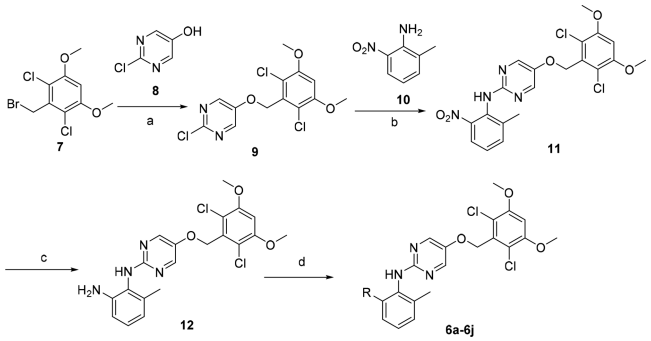


Figure 2. Design of compounds **6a−6j** based on lead **5** by replacing the acrylamide group with potential covalent warheads **I1−I10**.

Scheme 1. Synthesis of Compounds 6a−6j[a]



[a]Reagents and conditions: (a) K_2CO_3, Bu4N$^+$·I$^-$, DMF, 60 °C, 2.0 h. (b) $Pd(AcO)_2$, XantPhos, Cs_2CO_3, dioxane, 100 °C, overnight. (c) Fe, NH_4Cl, EtOH, H_2O, 70 °C, 1.0 h. (d) **6a**: 2-chloroethanesulfonyl chloride, Et_3N, dry DCM, 0 °C, 1.0 h; **6b**: cyanogen bromide, Cs_2O_3, dry THF, rt, 3.0 h; **6c**: chloroacetonitrile, K_2CO_3, NaI, MeCN, reflux, 3.0 h; **6d**: cyanoacetic acid, HATU, DIPEA, dry DMF, rt, overnight; **6e**: propargylbromide, K_2CO_3, DMF, rt, 4.0 h; **6f**: chloroacetyl chloride, Et_3N, dry DCM, 0 °C to rt, 1.0 h; **6g**: (i) glyceric acid, HATU, DIPEA, dry DMF, rt, overnight; (ii) methanesulfonyl chloride, pyridine, 0 °C, 1.0 h; (iii) K_2CO_3, MeOH, 0 °C to rt, 3.0 h; **6h**: 2-fluoroacrylic acid, HATU, DIPEA, dry DMF, rt, 1.0 h; **6i**: (i) 2-bromoethanol, K_2CO_3, THF, 65 °C, 16 h; (ii) PCC, dry DCM, 0 °C to rt, 1.0 h; **6j**: (i) cyanoacetic acid, HATU, DIPEA, dry DMF, rt, overnight; (ii) paraformaldehyde, piperidine, dry DCM, 0 °C to rt, 1.0 h.

under Lewis acid conditions gave N1-(5-((2,6-dichloro-3,5-dimethoxybenzyl)oxy)pyrimidin-2-yl)-6-methylbenzene-1,2-diamine (**12**). Finally, **12** was reacted with different warheads to produce the corresponding products **6a−6j** utilizing various reagents and conditions, as described.

The potential irreversible warheads **I1-I8** were initially incorporated into the 2-aminopyrimidine scaffold to give the corresponding compounds **6a−6h** (Table 1). Kinase inhibitory activities of all compounds against FGFR1−4 were evaluated by using a well-established FRET-based Z′-Lyte assay.[9] Compounds **6a** and **6h**, containing the vinylsulfonamide (**I1**) and α-fluoro acrylamide (**I8**) warheads, respectively, exhibited potent inhibitory activity against FGFR4 with biochemical IC_{50} values of 53 ± 18 and 45 ± 11 nM, respectively. Encouragingly, compound **6h** displayed excellent selectivity over FGFR1−3, whereas **6a** was 10-fold selective over its closest isoform (FGFR1). Compound **6g** containing the epoxy

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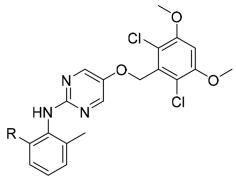
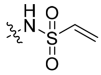
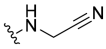
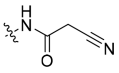
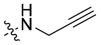
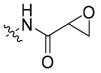
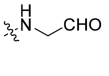
Table 1. FGFR1/2/3/4 Biochemical Kinase Inhibitory Activity of Compounds 6a−6j[a]



Compds	R	Kinase inhibition IC_{50} (μM)			
		FGFR4	FGFR1	FGFR2	FGFR3
6a		0.053± 0.018	0.52	2.71	1.23
6b		>10	>10	>10	>10
6c		>10	>10	>10	>10
6d		>10	>10	>10	>10
6e		>10	>10	>10	>10
6f		6.10	>10	>10	>10
6g		0.67±0. 19	>10	>10	>10
6h		0.045± 0.011	>10	>10	>10
6i		0.016± 0.004	>10	>10	>10
6j		>10	>10	>10	>10
5		0.072	>10	>10	>10
FGF401	-	0.01	>10	>10	>10

[a]Kinase activity assays were performed using a FRET-based Z′-Lyte biochemical assay.

amide warhead displayed moderate inhibitory activity against FGFR4 (IC_{50} = 0.67 ± 0.19 μM); however, compounds **6b−6f** demonstrated no inhibitory activity against FGFR4. We hypothesize that the relatively low level of conformational flexibility of Cys552 in the hinge region of FGFR4 limits the geometry and size of the electrophilic warheads that can be employed.[17] We further propose that warheads **I2−I5** and **I7** may not possess a suitable level of chemical reactivity, whereas the chloroacetamide warhead may be more suitable in the meta position of the aniline ring based on a recently published paper on a related scaffold.[18] The vinylsulfonamide and α-fluoro acrylamide warheads are suitable for binding with the conformationally restricted Cys552 due to their good rotational flexibility.

To elucidate the details of the interactions of compounds **6h** and **6j** with FGFR4, we attempted to generate both X-ray cocrystal structures. However, only the **6h**/FGFR4 complex was obtained. It was confirmed that **6h** occupies the ATP binding site of FGFR4 with a similar binding mode to that of lead **5** by forming a critical covalent bond with Cys552 (Figure 3A). The warhead α-fluoro acrylamide covalently reacts with

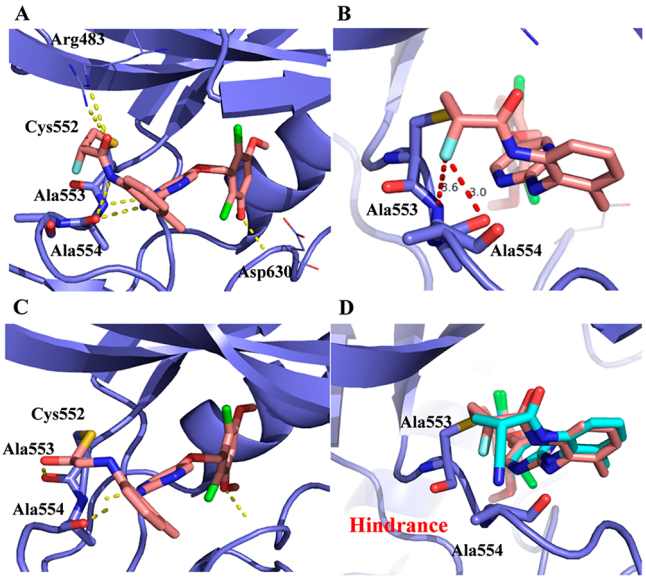


Figure 3. Binding mode analysis of compounds **6h**, **6j**, and **6k** with FGFR4. (A) X-ray crystal structure of FGFR4 with **6h** (PDB: 7DTZ). (B) Fluorine forms van der Waals interactions with Ala553. (C) Docking model of **6j** bound to FGFR4 based on the crystal structure of **5** bound to FGFR4 (PDB: 6NVH).[15] (D) Docking model of **6k** bound to FGFR4 with the **6h** crystal structure superimposed. The cyano group creates significant steric hindrance with Ala553.

the sulfhydryl group of Cys552, an interaction that drives the isoform selectivity. It is also noteworthy that the fluorine atom points toward Ala553 forming van der Waals interactions with this residue (Figure 3B). The 2-aminopyrimidine core forms two hydrogen bonds with the backbone amino and carbonyl groups of Ala553, respectively. The tetra-substituted phenyl group makes favorable van der Waals contacts with the hydrophobic back pocket and forms one hydrogen bond with Asp630.

Novartis researchers have suggested that the rapid resynthesis rate of FGFR4 in some HCC cell lines (<2 h) suggests that current acrylamide-bearing irreversible covalent inhibitors may not fully inhibit FGFR4 kinase signaling at a safe dose.[19] To achieve complete and continuous FGFR4 inhibition, they moved their focus to the development of reversible-covalent inhibitors. In the present study, the potential reversible-covalent derivatives **6i** and **6j** (based on the known reversible-covalent warheads **I9** and **I10**) were synthesized and evaluated (Table 1). The results indicate that only compound **6i** exhibits potent inhibitory activity against FGFR4, with an IC_{50} value of 16 ± 4 nM with excellent selectivity over FGFR1−3. Molecular docking studies indicated that **6i** displays a similar binding mode to that of **6h** (Figure 3C). The formyl group is proposed to form a covalent bond with Cys552 and an additional hydrogen bond with the carbonyl of Cys552, which contributes to the activity and selectivity. Compound **6j** totally lost inhibitory activity against FGFR4 (IC_{50} > 10 μM). The results demonstrate that the length of the warhead has an adverse influence on the inhibitory activity. Additionally, molecular modeling also suggests that **6j** with a cyanoacrylamide warhead lost inhibitory activity against FGFR4, presumably due to the steric hindrance between the cyano group and Ala553 (Figure 3D).

Protein mass spectrometry was performed to confirm the covalent binding of selected compounds. The molecular weight

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of FGFR4 is 34 652 Da, whereas the mass shifts after the incubation of compound **6h** with FGFR4 (Figure 4A). A

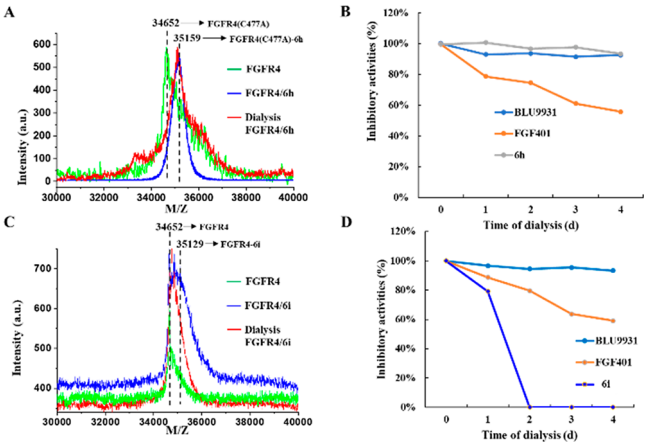

Figure 4. (A) MALDI-TOF MS determination of the FGFR4 and FGFR4/**6h** complex. (B) FGFR4/**6h** irreversible covalent binding determination using dialysis. (C) MALDI-TOF MS determination of FGFR4 and FGFR4/**6i** complex. (D) FGFR4/**6i** reversible-covalent binding determination using dialysis. Dialysis was performed after FGFR4/inhibitor incubation with activity recovery measured by FGFR4 kinase assay.

measured m/z of 35 159 Da is consistent with the sum of the molecular weight of **6h** and FGFR4, indicating the formation of a covalent adduct. In addition, **6h** exhibited negligible inhibition of FGFR4 C552A and FGFR4 C552S with IC_{50} > 10 μM (data not shown). A dialysis experiment was conducted to verify the covalent binding mode of **6h** with FGFR4. The inhibitors BLU9931 (irreversible covalent) and FGF401 (reversible covalent) were used as control compounds. As shown in Figure 4B, very little reversal of FGFR4 inhibition was observed with **6h** and BLU9931 following incubation and dialysis of the protein adduct over 4 days, indicating the permanent irreversible binding mode of **6h** and BLU9931 with FGFR4. By way of contrast, FGF401 demonstrated a ~40% return of FGFR4 kinase function over this period, consistent with its known reversible-covalent binding mode.

The same methodology was then applied to compound **6i**, which was found to display a reversible-covalent binding mode with FGFR4 (Figure 4C,D). Protein mass spectrometry indicated an m/z at 35 129 Da, consistent with the sum of the molecular weight of **6i** and FGFR4 following incubation. Additionally, the dialysis assay indicated the complete restoration of FGFR4 kinase activity after 2 days of dialysis, suggesting a significantly faster reverse reaction with Cys552 than that observed for FGF401.

Selected compounds were then assessed for their ability to inhibit the proliferation of cancer cell lines by sulforhodamine B (SRB) assay following a 5 day continuous exposure (Table 2). For this study, Hep3B HCC and MDA-MB-453 breast cancer cells were selected as examples of cell lines driven by high levels of wild-type FGFR4 and mutant $FGFR4^{Y367C}$ expression, respectively. To investigate the potential of the compounds to nonselectively inhibit the proliferation of cells, the FGFR4-independent NRAS mutant H1299 lung cancer cell line was utilized. Only the α-fluoro acrylamide derivative **6h** demonstrated significant antiproliferative activity selectively in the FGFR4-dependent cell lines. Whereas the FGFR4 potency of **6h** was four to six times less than that of the positive-control

Table 2. Antiproliferative Activity of Selected Compounds against Hep3B, MDA-MB-453, and H1299 Cancer Cells

	cellular inhibition IC_{50} (μM)		
compds	Hep3B	MDA-MB-453	H1299
6a	60.0	18.2	8.33
6g	59.3	24.8	133
6h	0.256	0.218	>20
6i	>100	66.1	78.4
BLU9931	0.058	0.039	1.03

compound BLU9931, it was encouragingly 20 times less cytotoxic to the negative-control H1299 cell line than BLU9931. Surprisingly, the reversible-covalent acetaldehyde amine **6i** failed to demonstrate potent antiproliferative activity in FGFR4-dependent cells, despite the potent inhibition of FGFR4 in the biochemical assay (Table 1). The reasons for the apparent discordance have yet to be fully elucidated, although we hypothesize that this result is consistent with the metabolic instability of the acetaldehyde in the cell culture, as observed for related aldehydes (in particular, in HCC cell lines) during the discovery of FGF401.

In summary, a series of 2-aminopyrimidine-based FGFR4 inhibitors incorporating different cysteine-targeting warheads were designed and synthesized. The representative compounds **6a**, **6g**, **6h**, and **6i** exhibited potent biochemical inhibitory activity against FGFR4, whereas **6g**, **6h**, and **6i**, in particular, were inactive against FGFR1/2/3. FGFR4 protein X-ray crystallography and MALDI-TOF studies demonstrated that compound **6h** bearing an α-fluoro acrylamide warhead binds to FGFR4 with an irreversible binding mode, whereas compound **6i** with an acetaldehyde amine warhead binds to FGFR4 with a reversible covalent binding mode. This study also indicates that the Cys552 binding site of FGFR4 shows high specificity for covalent inhibitors. Antiproliferative assays in cancer cell lines harboring dysregulated FGFR4 signaling demonstrated that **6h** possesses significant activity, whereas **6i** was unexpectedly inactive in cells. We propose that our study might provide some fundamental structural information for the rational design of new selective FGFR4 inhibitors. Further structural and pharmacokinetics-oriented optimization of **6h** is ongoing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00052.

Synthetic procedures for compounds **6a**−**6j**, materials and methods for biological assays, crystallization and structure determination, computational study, and the ^{1}H and ^{13}C NMR spectra of compounds **6a**−**6j** (PDF)

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Author Contributions

▽W.D., X.C., and K.J. contributed equally.

Notes

The authors declare no competing financial interest.

PDB ID 7DTZ contains the atomic coordinates and experimental data for the co-crystal structure of **6h** with FGFR4.

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