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2-Aminopyrimidine Derivatives as New Selective Fibroblast Growth Factor Receptor 4 (FGFR4) Inhibitors (Postprint)

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Abstract

A series of 2-aminopyrimidine derivatives were designed and synthesized as highly selective FGFR4 inhibi-tors. One of the most promising compounds 2n tightly bound FGFR4 with a Kd value of 3.3 nM and potently inhibited its enzymatic activity with an IC50 value of 2.6 nM, but completely spared FGFR1/2/3. The compound selectively suppressed proliferation of breast cancer cells harboring dysregulated FGFR4 signaling with an IC50 value of 0.38 M. Furthermore, 2n exhibited extraordinary target specificity in a Kinome-wide screen against 468 kinases, with S(35) and S(10) selectivity scores of 0.01 and 0.007 at 1.0 M, respectively.

Full Text

Preamble

2-Aminopyrimidine Derivatives as New Selective Fibroblast Growth Factor Receptor 4 (FGFR4) Inhibitors

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KEYWORDS: selective FGFR4 inhibitor, targeted therapy, breast cancer, hepatocellular carcinoma

ABSTRACT: A series of 2-aminopyrimidine derivatives were designed and synthesized as highly selective FGFR4 inhibitors. One of the most promising compounds, **2n**, tightly bound FGFR4 with a Kd value of 3.3 nM and potently inhibited its enzymatic activity with an IC50 value of 2.6 nM, while completely sparing FGFR1/2/3. The compound selectively suppressed proliferation of breast cancer cells harboring dysregulated FGFR4 signaling with an IC50 value of 0.38 M. Furthermore, **2n** exhibited extraordinary target specificity in a Kinome-wide screen against 468 kinases, with S(35) and S(10) selectivity scores of 0.01 and 0.007 at 1.0 M, respectively.

Introduction

The fibroblast growth factor receptor (FGFR) family comprises four functional members (FGFR1, FGFR2, FGFR3, and FGFR4), each consisting of a conserved extracellular ligand-binding domain, a single transmembrane region, and a cytosolic region with a split tyrosine kinase domain.[**?**] FGFRs serve as high-affinity receptors for fibroblast growth factors (FGFs), which include 18 members.[**?**] FGF/FGFR signaling is tightly regulated by ligand specificity and the temporal and spatial expression of signaling components, and is involved in many fundamental biological processes such as metabolism, embryonic development, and adult tissue homeostasis.[**?**, **?**] FGFR4 is mainly expressed in liver, lung, lymphoid, and breast tissues and specifically utilizes endocrine FGF19 as its ligand.[**?**, **?**] Upon binding to a complex formed by FGFR4 and its co-receptor, the transmembrane -Klotho protein, FGF19 promotes receptor dimerization, autophosphorylation, and activation of downstream signaling pathways that play significant roles in regulating bile acid homeostasis and maintaining glucose and protein metabolism.[**?**, **?**]

FGFR4 and/or FGF19 aberrations (amplification, gene fusion, or mutation) have recently been detected in a variety of human cancers. For instance, FGF19 is amplified in approximately 15% of breast cancers, and high FGF19 expression closely correlates with poor disease prognosis.[**?**, **?**] Various genetic alterations of FGFR4, including amplification, point mutation, and single nucleotide polymorphism, have also been detected in human breast tumor tissues.[**?**] Silencing FGF19/FGFR4 signaling by siRNA or using an anti-FGF19 antibody has been shown to potently suppress proliferation and induce apoptosis in triple-negative

breast cancer cells,[?, ?] offering promise for a disease setting that currently benefits little from available therapies.[?] A synergistic interaction has also been observed between FGFR4 signaling blockade and doxorubicin chemotherapy.[?, ?] Collectively, evidence suggests that FGF19/FGFR4 signaling acts as an oncogenic driver in a subset of hepatocellular carcinoma (HCC) patients. In an analysis of 281 HCC patients, approximately 48% of tumors expressed FGF19, which was associated with larger tumor size, more advanced stage, and early recurrence.[?] Additionally, about 30% of HCC samples displayed increased FGFR4 expression,[?] and 25% overexpressed -Klotho protein.[?] These data collectively indicate that targeting the FGF19/FGFR4 signaling pathway may provide a promising new strategy for managing defined subgroups of cancer patients.[?, ?]

A number of small-molecule FGFR inhibitors with different selectivity profiles have entered clinical investigation for managing FGFR-driven human cancers.[?] Unfortunately, hyperphosphatemia and tissue calcification are common "on-target" adverse effects for most drugs investigated,[?, ?] resulting from strong inhibitory activity against FGFR1 and FGFR3 that blocks FGF23-mediated signaling.[?] Given that FGFR family members share significant sequence homology in their kinase domains (Figure 1A [Figure 1: see original paper]), developing selective FGFR4 inhibitors over other family members represents a considerable challenge.

Most recently, BLU9931 (**1**, Figure 1B) was discovered as the first selective FGFR4 inhibitor. The structure-based design of this molecule involved covalent targeting of a unique Cysteine 552 (Cys552) residue in the hinge region of the receptor (Figure 1C).[?] The compound exhibited extraordinary target specificity and demonstrated significant anti-proliferative activity against a panel of human hepatocellular carcinoma cells with activated FGFR4 signaling. Moreover, it showed promising in vivo efficacy in several xenograft and patient-derived xenograft models of HCC.[?] More recently, BLU554, a structurally related derivative of inhibitor **1**, has advanced into early-stage clinical investigation (clinical trial ID NCT02508467), with clinical results eagerly awaited. Nevertheless, identifying new selective FGFR4 inhibitors remains highly valuable for anticancer drug discovery. Herein, we describe the design, synthesis, and biological evaluation of a series of 2-aminopyrimidine derivatives as new selective FGFR4 inhibitors.[?]

Design of New Selective FGFR4 Inhibitors

The X-ray co-crystal structure of compound **1** with FGFR4 revealed that the compound binds in the ATP-binding pocket of the receptor, forming a critical covalent bond with Cys552 (Figure 1C). The structure also showed that the C , C , C , and C atoms in this inhibitor adopt an almost coplanar configuration (Figure 1B/C), with no obvious direct interactions between phenyl ring A (Figure 1B) and the protein. It is well-established that the lowest-energy conformation of n-butane is an anti-configuration where the dihedral angle of the four

carbon atoms is approximately ±180° (Figure 1B).[**?**] Thus, an n-butane-like moiety might serve as a bioisostere of phenyl ring A to mimic the conformation of compound **1**.

Based on this hypothesis, we designed and synthesized a series of 2-aminopyrimidine derivatives (Figure 1B). Encouragingly, preliminary computational studies suggested that compound **2a**, which harbors a methyleneoxyl moiety to potentially mimic the conformation of the parent compound **1**, nicely accommodated the ATP-binding pocket of FGFR4 with a binding mode similar to that of compound **1** (Figure 1D). The warhead acrylamide is predicted to covalently react with the sulfhydryl side chain of Cys552, conferring paralog selectivity.[**?**] The 2-aminopyrimidine core forms two pairs of hydrogen bonds with the backbone amino and carbonyl groups of Ala553, respectively. The tetra-substituted phenyl group, which adopts an almost perpendicular orientation to the 2-aminopyrimidine core, makes favorable van der Waals contacts with the hydrophobic back pocket of the ATP-binding site, with one methoxyl group forming a hydrogen bond with the backbone carboxamide NH group of Asp630. Notably, compound **2a** possesses fewer aromatic rings and a higher Fsp^3 value than compound **1**, which may benefit its drug-like physicochemical properties.[**?**, **?**]

Synthesis of Compounds 2a-2z

The designed inhibitors were readily prepared using a Buchwald-Hartwig cross-coupling reaction as the key step (Scheme 1).[**?**] Briefly, commercially available 2-chloropyrimidin-5-ol was treated with various substituted benzyl bromides to give the corresponding intermediates **5** through a nucleophilic substitution reaction. Compounds **5** were coupled with various Boc-protected o-phenylenediamines under palladium catalysis to afford the Buchwald-Hartwig coupling products, which were deprotected to produce the arylamines **7**. Intermediates **7** were reacted with acryloyl chloride to yield the designed molecules **2a**, **2c-2g**, and **2k-2l** in good to moderate yields. Other designed inhibitors were synthesized using a similar protocol (Supporting Information).

Kinase Inhibitory Activities

The kinase inhibitory activities of the designed compounds against FGFR4 and family members FGFR1/2/3 were evaluated using a well-established FRET-based Z' -Lyte assay.[**?**] The first reported selective FGFR4 inhibitor **1** and the FGFR1/2/3 inhibitor FIIN-1[**?**] were used as positive controls to validate screening conditions. Under our assay conditions, compound **1** exhibited strong inhibitory potency against FGFR4 with an IC50 value of 6.0 nM and was significantly less potent against FGFR1/2/3, with our data comparing favorably to reported values.[**?**] In contrast, the pan-FGFR inhibitor FIIN-1 exhibited IC50 values of 3.6, 2.3, 6.0, and 261.3 nM against FGFR1, FGFR2, FGFR3, and FGFR4, respectively.

We were delighted to find that our first designed compound **2a** potently suppressed FGFR4 enzymatic activity with an IC50 value of 11.1 nM, only 2-fold less potent than positive control **1**, while completely sparing FGFR1/2/3 (IC50 > 10,000 nM) (Table 1). Investigation also suggested that the methyleneoxyl linker in **2a** could be replaced by a methyleneamino moiety (**2b**) with slightly decreased FGFR4 suppression (IC50 = 43.4 nM) while maintaining paralog selectivity.

Compound **2a** harbored a methyl substitution at the 3' -position, adopted from the original parent molecule **1** and considered important for selective FGFR4 inhibition. We first investigated the potential impact of methyl substituent position on paralog-sparing FGFR4 inhibition. The substituent position indeed greatly influenced target inhibitory potency and paralog selectivity. When the methyl group was shifted to the 6' -position, the resulting molecule **2c** demonstrated complete loss of all FGFR inhibitory activity. The 5'-methyl substituted compound **2d** exhibited slightly increased FGFR4 inhibitory potency (IC50 = 7.5 nM) but decreased selectivity due to increased FGFR1 inhibition. In contrast, a 4' -methyl substituent (**2e**) resulted in slight decrease in FGFR4 inhibitory potency and similar FGFR1 inhibition as observed in the 5' -position (**2d**). Although removal of the methyl group barely affected FGFR4 inhibition potency, the resulting molecule **2f** displayed obvious suppression against both FGFR1 and FGFR2 (IC50 = 431.0 and 311.9 nM, respectively), suggesting significant loss of target specificity. These results collectively indicate that 3' -substitution is an optimal strategy for designing new selective FGFR4 inhibitors.

Further investigation revealed that the 3' -position tolerates a variety of substituents, including ethyl (**2g**), methoxyl (**2h**), chloro (**2l**), and trifluoromethyl (**2m**) moieties, which demonstrated potent and paralog-selective FGFR4 inhibition. Encouragingly, the 3' -chloro substituted analogue **2l** exhibited markedly improved FGFR4 inhibition potency (IC50 = 3.8 nM) while retaining excellent selectivity against FGFR1/2/3 at 10,000 nM. Not surprisingly, the 3' -fluoro substituted compound **2k** displayed a similar potency and selectivity profile to the 3' -unsubstituted molecule **2f**. Notably, increasing the size of the 3' -alkoxy group resulted in stepwise loss of FGFR4 inhibitory potency (compare **2h**, **2i**, and **2j**).

Our investigation suggested that substitution at the 5' -position could retain strong FGFR4 inhibition. Therefore, several 3',5'-disubstituted compounds (**2n**, **2o**, **2p**) were designed and synthesized, displaying potent and selective FGFR4 suppression. Particularly, the 3'-chloro-5'-fluoro analogue **2n** exhibited the best FGFR4 inhibitory activity (IC50 = 2.6 nM), 2-fold more potent than compound **1**. Compound **2n** also displayed obviously improved paralog selectivity over reported inhibitor **1**.

Further investigation revealed that the acrylamide warhead was crucial for potent FGFR4 inhibition. Replacement of the acrylamide with an , -saturated propionamide moiety (**2q**) completely abolished FGFR inhibition. It is gen-

erally accepted that a benzyloxy group possesses potential metabolic liability, and introducing steric hindrance is a feasible strategy to minimize oxidative metabolism and improve metabolic stability. Based on this rationale, compound **2r**, bearing a methyl group on the benzyloxyl linker, was designed and synthesized. Disappointingly, this modification caused a dramatic 220-fold loss in potency.

Modification of the tetra-substituted phenyl moiety, predicted to approach the hydrophobic back pocket of the ATP-binding site, showed that tetra-substitution was critical for strong FGFR4 inhibition. Removal of one or more substituents caused significant potency loss against FGFR4. For instance, removal of a methoxyl group (**2s**) resulted in 6.3-fold potency loss, whereas deletion of a chloro group (**2t**) reduced potency 3-fold. Other chloro- or methoxyl-deleted compounds (**2u-2z**) displayed 6.0- to 122-fold potency losses. Thus, compounds **2a**, **2h**, **2l**, **2o**, and **2n** stood out as representatives for further investigation due to their strong FGFR4 inhibitory potency and extraordinary paralog specificity.

Cellular Validation of Compound 2n

Using compound **2n** as an example, we further validated the strong FGFR4 inhibition by investigating its effects on FGFR4 activation and downstream signaling partners in MDA-MB-453 cells harboring an activating FGFR4^{Y367C} mutation (Figure 2A).[**?**] The compound diminished FGFR4 phosphorylation and inhibited activation of downstream FRS2, ERK1/2, and AKT in a dose-dependent manner, while total protein levels remained unchanged as determined by western blot analysis. Phosphorylation of FGFR4, ERK1/2, and FRS2 was almost completely abolished after treatment with 0.03 M of **2n**, although its effect on p-AKT was less pronounced.

The binding affinity of **2n** for FGFR4 was determined using an active-site-dependent competition binding assay (DiscoveRx Corporation, San Diego, USA). Compound **2n** tightly bound to the ATP-binding site of the kinase with a binding constant (Kd) of 3.3 nM, validating its strong FGFR4 inhibition. We further profiled the target specificity of this compound against a panel of 468 kinases (including 403 non-mutated kinases) using the DiscoveRx screening platform at 1.0 M, approximately 300 times its Kd value for FGFR4. The results revealed that **2n** demonstrated impressive target specificity with S(10) and S(35) selectivity scores of 0.007 and 0.01 at 1.0 M, respectively (Table S2). For instance, **2n** showed almost 100% competition (99.5% inhibition, ctrl% = 0.5) with FGFR4 at 1.0 M and only obvious binding to a very small fraction of kinases investigated. The main "off-target" hits included calcium/calmodulin-dependent protein kinase ID (CAMK1D, 94.7% inhibition), G-protein-coupled receptor kinase 4 (GRK4, 92.8% inhibition), and SRSF protein kinase 3 (SPRK3, 88% inhibition).

Antiproliferative Activities

The growth inhibitory effects of compounds **2a**, **2h**, **2l**, **2o**, and **2n** were evaluated against a panel of breast cancer cell lines (Table 2). MDA-MB-453 breast cancer cells predominantly express a mutated form of FGFR4, FGFR4^{Y367C}, which causes spontaneous receptor dimerization and constitutive activation in a ligand-independent manner.[**?**] Moreover, FGFR4^{Y367C} has been identified as an important driver gene for MDA-MB-453 cell proliferation. In contrast, MCF-7 and MDA-MB-231 breast cancer cells harbor low FGFR4 levels and serve as negative control models to investigate inhibitor target specificity.[**?**]

Almost all selected FGFR4 inhibitors exhibited promising antiproliferative activity against MDA-MB-453 breast cancer cells with potencies similar to inhibitor **1**. However, none displayed obvious cell growth inhibition against MCF-7 and MDA-MB-231 cells expressing low FGFR4 levels. For instance, compound **2n** potently inhibited MDA-MB-453 proliferation with an IC50 of 0.38 M, comparable to compound **1**, but was unable to suppress MCF-7 and MDA-MB-231 growth at 10 M. These data further support the strong and selective antagonism of the new inhibitors against FGFR4. Notably, all new inhibitors appear to display better target specificity than the original compound **1** in cell-based assays, consistent with biochemical kinase inhibition data. Further investigation demonstrated that **2n** dose-dependently induced apoptosis in MDA-MB-453 cells (Figure S2).

Conclusion

In summary, we designed and synthesized a series of 2-aminopyrimidine derivatives as highly selective FGFR4 inhibitors. These compounds potently suppressed FGFR4 activity with low nanomolar IC50 values and excellent isotype selectivity. One of the most promising compounds, **2n**, tightly bound FGFR4 with a Kd of 3.3 nM and potently inhibited its enzymatic activity with an IC50 of 2.6 nM while completely sparing FGFR1/2/3. The compound selectively suppressed proliferation of breast cancer cells harboring dysregulated FGFR4 signaling with an IC50 of 0.38 M. Furthermore, it exhibited extraordinary target specificity in a Kinome-wide screen against 468 kinases, with S(10) and S(35) selectivity scores of 0.007 and 0.01 at 1.0 M, respectively. Compound **2n** may serve as a new lead compound for future anticancer drug discovery. Further structural optimization and in vivo antitumor efficacy investigation are ongoing.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures for synthesis, ^1H NMR and ^13C NMR for final compounds, kinase selectivity, and details of in vitro assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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