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Synthesis and biological study of 4-aminopyrimidine-5-carboxaldehyde oximes as antiproliferative VEGFR-2 inhibitors

Shenlin Huang,[a,*] Ronghua Li,[a] Peter J. Connolly,[a] Guozhang Xu,[b] Michael D. Gaul,[b] Stuart L. Emanuel,[a] Kenneth R. LaMontagne[a] and Lee M. Greenberger[a]

[a]*Johnson and Johnson Pharmaceutical Research and Development, L.L.C, 1000 Route 202, Raritan, NJ 08869, USA*
[b]*Johnson and Johnson Pharmaceutical Research and Development, L.L.C, 8 Clarke Drive, Cranbury, NJ 08512, USA*

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Abstract—A novel 4-aminopyrimidine-5-carboxaldehyde oxime scaffold with inhibitory activity against VEGFR-2 kinase has been identified. With a 4-fluoro-2-methylindol-5-yloxy group at the 6-position and alkyl groups as the oxime side chains, many analogues showed good potency for VEGFR-2. This series also exhibited antiproliferative activity against cancer cells, causing cell accumulation at the G2/M phase of the cell cycle and preventing cells from entering mitosis. Described here are the chemistry, structure–activity relationships (SAR), and biological testing for this series.

Angiogenesis, the formation of new capillaries from the endothelial cells of preexisting vascular networks, plays a vital role in tumor growth. Solid tumors cannot grow beyond a few millimeters until they develop new collateral blood vessels to provide oxygen and nutrients.[1] Although several growth factors play important roles in angiogenesis, vascular endothelial growth factor (VEGF) and its cognate tyrosine kinase receptor (VEGFR-2) are of particular interest because of their involvement in multiple processes of angiogenesis, including vascular permeability, endothelial cell activation, proliferation, and migration.[2] Inhibition of VEGF activity or VEGFR-2 kinase has been shown to suppress tumor angiogenesis and tumor growth in tumor xenograft studies. The use of antiangiogenic agents for cancer therapy has been the focus of intensive pharmaceutical research. FDA approval of the anti-VEGF antibody bevacizumab for the treatment of colorectal cancer provides valuable proof-of-concept in a clinical setting.[3] Recently, two small molecule drugs that inhibit VEGFR-2 kinase, sorafenib (BAY-43-9006)[4] and sunitinib (SU-11248),[5] were approved by the FDA for renal and/or gastrointestinal cancer. Numerous other small molecules have progressed to the clinical evaluation stage.[6,7]

Our effort to develop small molecular ATP-competitive VEGFR-2 inhibitors as cancer therapeutics led to the discovery of 4-aminopyrimidine-5-carboxaldehyde oximes having potent VEGFR-2 inhibitory activity. This novel series also shows antiproliferative activity against cancer cells. Combination of antiangiogenesis and antiproliferation could enhance in vivo antitumor efficacy, especially for the therapy of advanced cancer.[8] Described here are the chemistry, structure–activity relationships (SAR), and biological testing for this series.

As shown in Scheme 1, the general chemistry for analogue synthesis started with 4-amino-6-chloropyrimidine-5-carboxaldehyde (**1**).[9] Displacement of the 6-chloride with R^1OH in the presence of cesium carbonate resulted in compound **2**, which was reacted with hydroxylamine (HCl salt) in DMSO to generate oxime **3**. This condensation step proceeded in quantitative yield with the *E*-isomer as the sole product. Alkylation of the hydroxyl group of compound **3** with an alkyl bromide in DMF gave final compound **4**. Slight warming of reaction mixture was required for the formation of some analogues. Alternatively, **4** was prepared by reacting aldehyde **2** directly with the appropriate *O*-alkylated hydroxylamine HCl salt in DMSO. Using this highly efficient chemistry, many analogues were synthesized.

The oxime analogues were tested for VEGFR-2 kinase inhibition and were also screened for antiproliferative activity against three cancer cell lines, HeLa (cervical

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* Corresponding author. Tel.: +1 908 392 0158.; e-mail: shenlinhuang@hotmail.com

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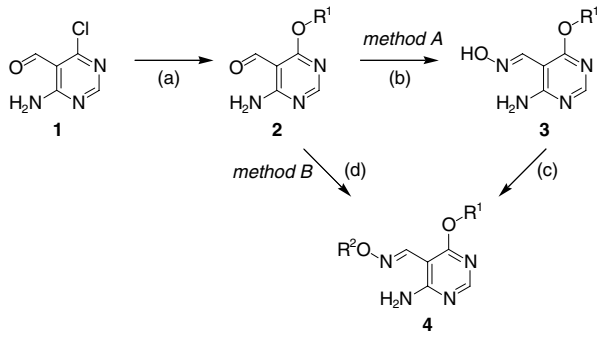


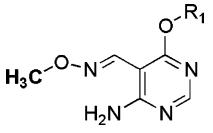
Scheme 1. Synthesis of 4-aminopyrimidine-5-carboxaldehyde oximes. Reagents and conditions: (a) R^1OH, Cs_2CO_3, DMSO, method A (analogues **14**–**19**, **25**, **26**, and **29**); (b) $HONH_2{\cdot}HCl$, DMSO; (c) R^2Br, K_2CO_3, DMF, method B (analogues **5**–**13**, **20**–**24**, and **28**); (d) $R^2ONH_2{\cdot}HCl$, DMSO.

adenocarcinoma), HCT116 (colon carcinoma), and A375 (malignant melanoma). The kinase and cell data are summarized in Tables 1 and 2. Our initial SAR study was focused on the aryl ether group, R^1. With R^1 as a quinolin-6-yl or quinolin-7-yl group, only weak potency for VEGFR-2 was achieved. Analogues **5** and **6** also showed poor antiproliferative activity. Compound **7** with an unsubstituted indol-5-yl group showed moderate inhibition of VEGFR-2. To our surprise, adding one methyl group to the N-1 or C-3 position of the indol-5-yl ring (**8** and **10**, respectively) was detrimental to VEGFR-2 potency but beneficial to cellular inhibition. This observation suggests that there are different structural requirements for VEGFR-2 kinase inhibition and cellular antiproliferative activity. Shifting the methyl group to the C-2 position of the indol-5-yl ring resulted in derivative **9** that was not only potent against VEGFR-2 kinase but also potent in cells. Adding one fluoro group to the C-4 or C-6 position of the indol-5-yl ring (**11** and **12**, respectively) also increased the potency for both VEGFR-2 kinase and cells. The combination of a methyl group at C-2 and fluoro group at the C-4 position of the indol-5-yl group was additive, generating **13**, the most potent derivative of the series with an IC_{50} of 42 nM against VEGFR-2 and 27–36 nM IC_{50} values in cells.

After optimization of the R^1group, a SAR study for the oxime side chain (R^2) was undertaken. As shown in Table 2, when R^2 was an ethyl, propyl, allyl, or propargyl group (**13**, **14**, **16** or **17**), high potency for both VEGFR-2 and cell proliferation was achieved. However, a bulkier butyl group (**15**) was less tolerated at this position, resulting in IC_{50} values of 115 nM for VEGFR-2 and 1.0 μM for HCT116 cells. A cyclohexylmethyl group (**18**) was also detrimental to activity. On the other hand, a benzyl group (**19**) maintained decent cell potency although it also lowered kinase potency. This phenomenon is consistent with previous observation that SARs for VEGFR-2 and cancer cells are different. Solubilizing amino groups such as 2-(*N*-methylamino)ethyl, 2-(*N*,*N*-dimethylamino)ethyl, 2-(pyrolidin-1-yl)ethyl, or 3-(*N*,*N*-dimethylamino)propyl (**20**–**22**, **24**) were slightly detrimental to VEGFR-2 potency but more harmful to cell activity, indicating that the R^2 group might fit in a lipophilic binding pocket. The exception is the (morpholin-4-yl)ethyl group (**23**), which showed excellent kinase potency and reasonable cellular potency. Antiproliferative activity seems to correlate with lipophilicity of the oxime side chain and inversely with basicity of the pendant amino group. Among the amino analogues, the least basic group (morpholine) conferred the highest cell potency, suggesting that the more basic analogues do not penetrate the cell membrane well. Unsubstituted analogue **27** had an IC_{50} of 33 nM for VEGFR-2. With R^2 as hydroxyalkyl or alkoxyalkyl groups, compounds **25**, **26**, **28**, and **29** exhibited similar potency as compound **27**, though these analogues were less potent in cells compared to those with simple alkyl R^2 groups.

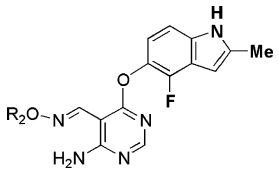
Flow cytometric analysis was used to study the cell cycle effect of compound **16** on human HeLa tumor cells. In this assay cells were stained with propidium iodide and analyzed by fluorescence; then DNA content and cell cycle phase distribution were estimated. As shown in Figure 1A, untreated HeLa cells showed a normal cell cycle phase distribution with 59% in G1 phase, 26% in S phase, and 14% in G2 phase. Treatment of HeLa cells with 62.5 nM of compound **16** resulted in an accumulation of cells at G2 phase with 0.03% in S phase and >99% in G2 phase (Fig. 1B). The cells were prevented

Table 1. Kinase and cellular antiproliferative activity for analogues **5**–**12**



Compound	R^2	VEGFR-2 inhibition IC_{50} (μM)	Antiproliferative IC_{50} (μM)		
			HeLa	HCT116	A375
5	Quinolin-6-yl	0.700	3.430	2.868	1.825
6	Quinolin-7-yl	1.588	1.817	2.624	0.886
7	Indol-5-yl	0.276	1.871	3.522	0.783
8	1-Methylindol-5-yl	1.189	0.375	0.749	0.326
9	2-Methylindol-5-yl	0.146	0.388	0.386	0.356
10	3-Methylindol-5-yl	1.038	0.306	0.385	0.132
11	4-Fluoroindol-5-yl	0.117	0.401	0.365	0.492
12	6-Fluoroindol-5-yl	0.121	0.382	0.422	0.327

Table 2. Kinase and cellular antiproliferative activity for analogues **13**–**29**



Compound	R^2	VEGFR-2 inhibition IC_{50} (μM)	Antiproliferative IC_{50} (μM)		
			HeLa	HCT116	A375
13	CH_3CH_2	0.042	0.032	0.036	0.027
14	$CH_3CH_2CH_2$	0.043	0.040	0.030	0.028
15	$CH_3CH_2CH_2CH_2$	0.115	0.607	1.004	0.446
16	CH_2=$CHCH_2$	0.052	0.042	0.040	0.021
17	Propargyl	0.052	0.028	0.028	0.030
18	Cyclohexyl-CH_2	0.413	22.66	16.06	4.924
19	Bn	0.201	0.266	0.311	0.062
20	$CH_3NHCH_2CH_2$	0.093	2.008	3.007	0.345
21	$(CH_3)_2NCH_2CH_2$	0.187	3.227	4.246	0.501
22	(Pyrrolidin-1-yl)-CH_2CH_2	0.214	4.221	4.61	2.156
23	(Morpholin-4-yl)-CH_2CH_2	0.039	0.699	1.164	0.339
24	$(CH_3)_2NCH_2CH_2CH_2$	0.101	4.334	4.287	1.599
25	$CH_3OCH_2CH_2$	0.024	0.308	0.387	0.233
26	$CH_3OCH_2CH_2CH_2$	0.062	0.346	0.438	0.300
27	H	0.033	0.472	1.136	0.492
28	$HOCH_2CH_2$	0.044	0.406	0.323	0.372
29	$HOCH_2CH_2CH_2$	0.011	0.362	0.452	0.336

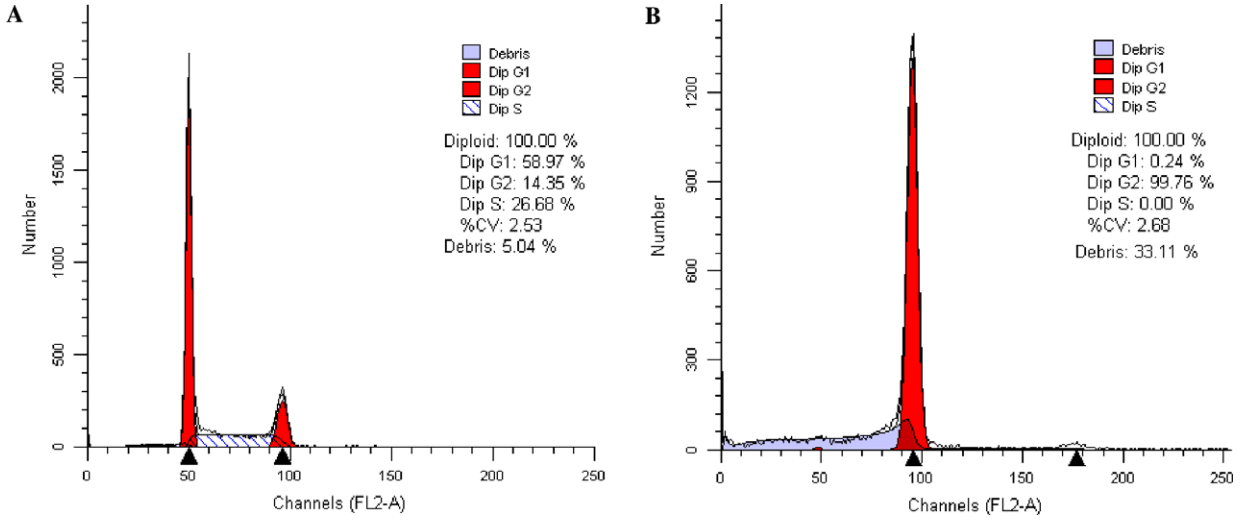


Figure 1. (A) Flow cytometric analysis of control human HeLa tumor cells. (B) Flow cytometric analysis of human HeLa tumor cells treated with 62.5 nM of compound **16**.

from entering mitosis. The cell cycle effect of this series might contribute to its antiproliferative activity.

Selected compounds **13** and **25** were screened against a panel of 100 kinases at the concentration of 3 μM in the presence 2 μM ATP.[10] Both compounds were highly selective for VEGFR-2 compared to other VEGFR family members VEGFR-1 and VEGFR-3. They showed very weak (<30%) inhibition of other angiogenesis-related kinases including FGFR, PDGFR, and Tie2. Compounds **13** and **25** displayed >30% inhibition on only 5 and 1 kinases in the panel, respectively, and did not inhibit cell cycle kinases like that are implicated in G2/M blockade of the cell cycle (i.e., CDK1). This high selectivity could reduce potential in vivo off-target side effects and offer an advantage over current clinically tested VEGFR-2 compounds.[6,7]

To investigate the biological mechanism for antiproliferative activity, compound **16** was tested in tubulin assembly assay. No inhibition of microtubule polymerization was observed even with 10 μM of compound **16**. Therefore,

its cellular activity is due to other unknown mechanisms, instead of tubulin inhibition.

In summary, a novel series of 4-aminopyrimidine-5-carboxaldehyde oxime having activity against VEGFR-2 kinase has been identified. The best potency was achieved with 4-fluoro-2-methylindol-5-yloxy at the 6-position. On the oxime side chain, lipophilic alkyl groups were preferred over hydrophilic amino side chains. This scaffold had shown antiproliferative activity against several cancer cell lines and caused accumulation of HeLa cells at the G2/M phase. It exhibited high selectivity for VEGFR-2 kinase and showed no antimicrotubule activity. Future studies will be focused on in vivo antitumor efficacy in this series.

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