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Novel 4-amino-furo[2,3-*d*]pyrimidines as Tie-2 and VEGFR2 dual inhibitors

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Abstract—A novel class of furo[2,3-*d*]pyrimidines has been discovered as potent dual inhibitors of Tie-2 and VEGFR2 receptor tyrosine kinases (TK) and a diarylurea moiety at 5-position shows remarkably enhanced activity against both enzymes. One of the most active compounds, 4-amino-3-(4-((2-fluoro-5-(trifluoromethyl)phenyl)amino-carbonylamino)phenyl)-2-(4-methoxyphenyl)furo[2,3-*d*]pyrimidine (**7k**) is <3 nM on both TK receptors and the activity is rationalized based on the X-ray crystal structure.

Angiogenesis, the formation of new blood vessels by capillary sprouting from pre-existing vasculature, has been shown to be involved in many diseases such as diabetic retinopathy, psoriasis, rheumatoid arthritis, and cancer. In particular, it is widely accepted that growth and metastasis of solid tumors is dependent on angiogenesis.[1] Out of the many factors shown to be involved in angiogenesis, vascular endothelial growth factor (VEGF) and angiopoietins are of particular interest as these are specific factors for endothelial cells and expression of their receptors is restricted to these cells.[2] VEGF and angiopoietins have been thought to play complementary and coordinated roles in vascular development.[3] During development, VEGF and its receptor VEGFR2 play crucial roles in vessel sprouting and new vessel initiation in early stages of angiogenesis through induction of proliferation, migration, and survival of endothelial cells.[4] Ang1 and its receptor Tie-2 play an important role in stabilizing the immature endothelial cell network, attracting pericytes, and maintaining biochemical interactions and vessel integrity, which are thought to be implemented in secondary stages of blood vessel formation.[5]

The development of a dual inhibitor of VEGFR2 and Tie-2 would be expected to demonstrate synergistic effects through inhibition of both critical stages of blood vessel formation and offers the potential for new approaches for the discovery of anti-angiogenesis agents.[6] Herein, we wish to report the synthesis and biological activity of 4-amino[2,3-*d*]furopyrimidines and the discovery that diarylurea derivatives show VEGFR2/Tie-2 dual inhibitory activity both at the enzyme and cell levels.[7] We will also describe an X-ray crystal structure of an inhibitor complexed with VEGFR2 to rationalize the enzyme potency of the 4-amino[2,3-*d*]furopyrimidines.

From the results of a focused screening effort, 5,6-diaryl-4-amino[2,3-*d*] furopyrimidines, such as **1** (IC_{50} = 1.25 μM and 1 μM vs VEGFR2 and Tie-2, respectively), were identified as compounds with moderate inhibitory activity against VEGFR2 and Tie-2.[8] According to a pharmacophore model for ATP competitive kinase inhibitors (see Fig. 1), we can speculate that (1) the aminopyrimidine moiety of the furopyrimidine core binds to the hinge region through hydrogen-bond donor–acceptor interactions in a similar manner to the N1 and N6 in the adenine base of ATP; (2) the 5-aryl moiety would fill a hydrophobic region not generally

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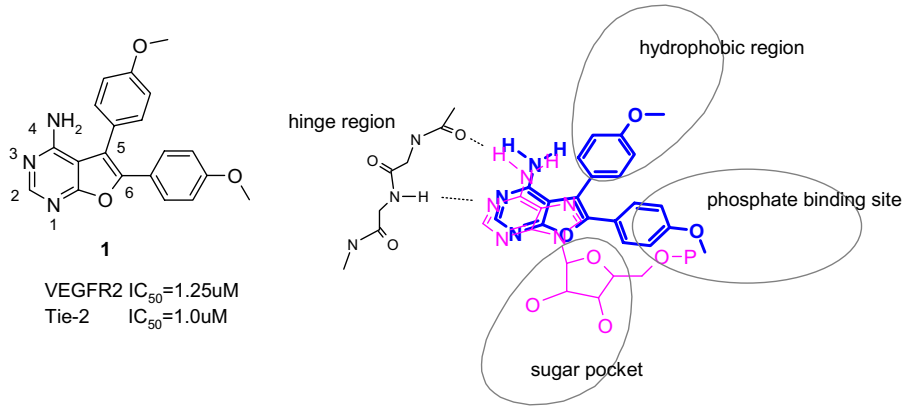
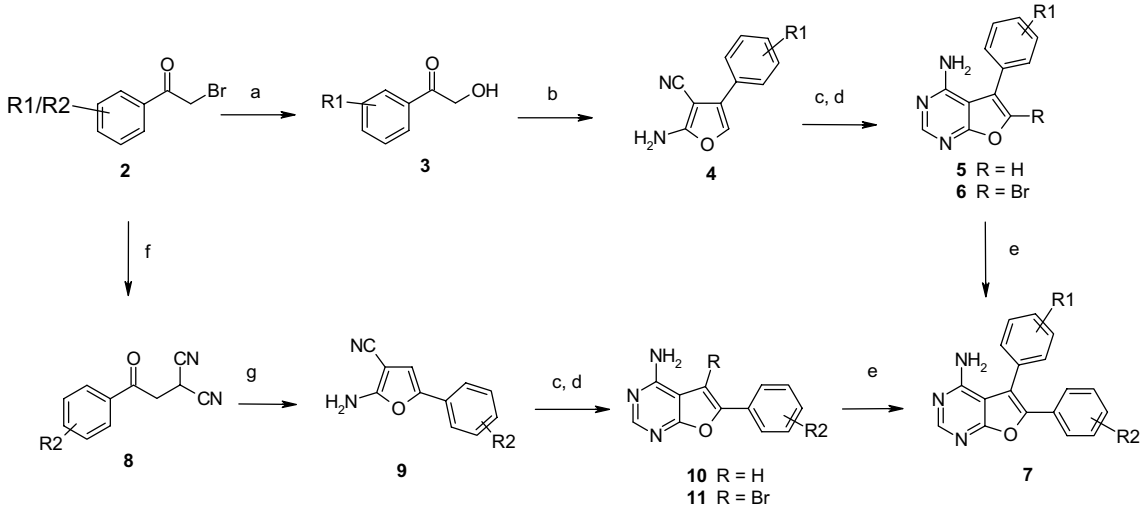


Figure 1. Original hit compound **1** and proposed binding mode in protein kinase pharmacophore with ATP (**1**, blue; ATP, magenta).

occupied by ATP in most protein kinases; (3) the 6-aryl group would be exposed to the phosphate binding region. Based on these hypotheses, we focused on the derivatization of both the 5- and 6-position, in order to improve the potency.

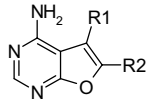
Synthesis of 5,6-diaryl-furo-4-amino[2,3-*d*]pyrimidines **7** for exploring the structure–activity relationship (SAR) of the 5- and 6-positions was accomplished using procedures illustrated in Scheme 1.[9] In the procedure to derivatize the 6-position, 1-aryl-2-bromoethanone (**2**) was converted to 1-aryl-2-hydroxyethanone (**3**) using potassium formate and aqueous sodium bicarbonate, which was treated with malononitrile in the presence of diethylamine to give furan **4**. Cyclization with formamide led to furo[2,3-*d*]pyrimidine **5**. Bromination with NBS gave **6** and a palladium mediated coupling with a variety of boronic acids resulted in derivatives of **7**. In another procedure focused on derivatizing the 5-position, 1-aryl-3,3-dicyanopropan-1-one (**8**) was prepared from **2** and malononitrile using NaOEt as the base. A double cyclization, first under acidic conditions followed by pyrimidine formation using formamide afforded **10**. The 5-bromo intermediate **11** was prepared by bromination of 6-aryl-furo[2,3-*d*]pyrimidine **10** with NBS. Intermediates **11** were coupled under palladium catalyzed conditions with aryl boronic acids leading to **7**.

The enzyme inhibitory activities are summarized in Table 1. As indicated in the data, mono aryl derivatives **5a** and **10a** show reduced activity compared to that of **1**. Apparently, 5, 6-diaryl moieties are important for potent enzyme activity. With R1 retained as the 4-methoxyphenyl substituent, changes to determine the SAR at the 6-position were investigated. Compounds with hydrogen-bond donor–acceptors such as 3-carboxamide **7b** modestly improved the enzyme inhibitory activity compared with that of the initial hit compound **1**. However, a *meta*-methanesulfonamide **7d** introduced a modest 3-fold improvement in Tie-2 activity and a more dramatic 20-fold improvement in VEGFR2 activity. When R2 is retained as the 4-methoxyphenyl group and the 3-methanesulfonamide substituent, the 5-position *N*,*N*-dimethylaminophenyl and biphenyl derivatives



Scheme 1. Reagents and conditions: (a) potassium formate, $NaHCO_3$, H_2O–EtOH, 40 °C, 90%; (b) malononitrile, Et_2NH, DMF, rt, 87%; (c) formamide, reflux, 69–74%; (d) NBS, CCl_4, 50–88%; (e) aryl boronic acid/ester, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C; (f) malononitrile, NaOEt, EtOH, rt, 89%; (g) acetic acid, concd HCl, rt, 56%. Yields are shown for **7** where R1 = R2 = 4-OMe.

Table 1. Tie-2 and VEGFR2 kinase enzyme inhibition of 4-NH_2 furo[2,3-*d*]pyrimidines[5]



Compound	R1	R2	Tie-2 (μM)	VEGFR2 (μM)
1	4-OMe-phenyl	4-OMe-phenyl	1.0	1.12
5a	4-OMe-phenyl	H	>20	5.5
10a	H	4-OMe-phenyl	>20	>17
7a	4-OMe-phenyl	3,4-Cl_2-phenyl	1.78	>20
7b	4-OMe-phenyl	3-$CONH_2$-phenyl	0.54	0.18
7c	4-OMe-phenyl	3-$CONMe_2$-phenyl	1.48	ND
7d	4-OMe-phenyl	3-$NHSO_2$Me-phenyl	0.30	0.065
7e	4-NMe_2-phenyl	4-OMe-phenyl	0.60	0.35
7f	4-Biphenyl	3-$NHSO_2$Me-phenyl	0.19	0.036
7g	3-NHAc-phenyl	4-OMe-phenyl	>20	ND

IC_{50} value are generated by measuring inhibition of peptide substrate added to enzyme reaction in homologous time-resolved fluorescence format (HTRF). ND = not determined.

(**7e** and **f**, respectively), increased enzyme potency. We envision that this is due to hydrophobic interactions of the biphenyl group at the 5-position, which is expected to project into the hydrophobic region that is not fully occupied by the 4-methoxyphenyl group. Additionally, interactions of the dimethylamino moiety with Lys866 (see Fig. 3) may have also improved the activity. Based on the assumption that a large hydrophobic pocket exists into which the 5-position of the furopyrimidine core projects and that interactions with Lys866 would be expected, additional 5-position functionalized analogues were evaluated and are listed in Table 2. Based on this hypothesis, compound **7j** wherein arylurea is substituted at the *para* position was prepared and it had remarkably enhanced Tie-2 and VEGFR2 enzyme activities. The 2-fluoro-5-trifluoromethyl phenyl-urea **7k** is the most active analogue and enhanced potency by approximately 15-fold against Tie-2 and 20-fold against VEGFR2, compared with the unsubstituted phenyl-urea **7j**. Amide and sulfonamide derivatives (**7h** and **i**) decreased potency.

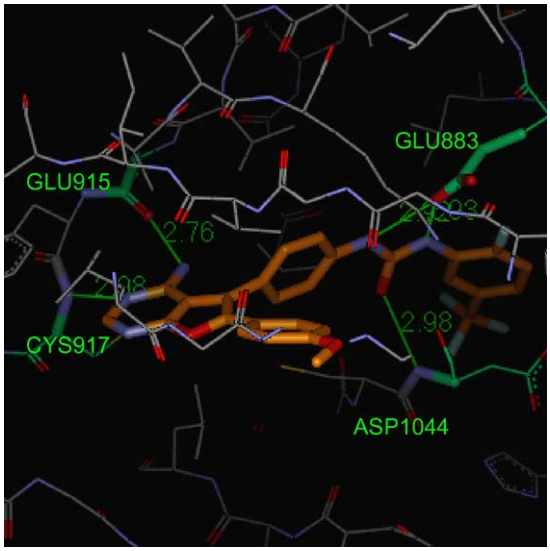
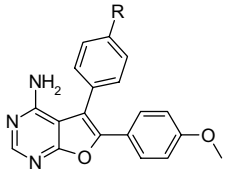


Figure 2. X-ray structure of compound **7k** (orange) complexed with VEGFR2.

The crystal structure of VEGFR2 with **7k** was determined at high resolution, as shown in Figure 2.[10] The NH and CO motifs of the urea form interactions with the backbone of Asp1044 and the carboxylic acid residue of Glu883, respectively. The NH_2 and nitrogen of

Table 2. Tie-2 and VEGFR2 kinase enzyme inhibition of 4-NH_2 furo[2,3-*d*]pyrimidines



Compound	R	Tie-2 (μM)	VEGFR2 (μM)
7h	–NHCO-(3-fluorophenyl)	0.501	ND
7i	–$NHSO_2$-(3-chlorophenyl)	0.851	ND
7j	–NHCONH-phenyl	0.028	0.062
7k	–NHCONH-(2-fluoro-5-trifluorophenyl)	0.002	0.003
7l	–NHCONH-(4-chlorophenyl)	0.022	ND
7m	–NHCONH-cyclohexyl	0.059	0.186
7n	–$NHCONH_2$	0.275	0.058

the aminopyrimidine form interactions with Glu915 and Cys917. Taking into consideration the fact that the enzyme potency of **7m** and **n** decreased because of the lack of a terminal aryl group, the binding potency therefore relies on strong interactions of the 2-fluoro-5-trifluoromethyl phenyl moiety with the hydrophobic area which exists beyond the hydrophilic region flanked by Lys866, Glu883, and the NH of Asp1044. As shown in Figure 3, the terminal aryl ring and its substitutions are accommodated into this hydrophobic area composed of residues Ile886, Leu887, Ile890, Val896, and Leu1017. Additionally the urea NH and carbonyl groups assisted in stabilizing the molecule by interaction with the Lys866-Glu883 salt bridge and the NH of Asp1044. In a manner similar to the binding in VEGFR2, the terminal aryl group of the urea moiety in **7k** would be expected to reside in the hydrophobic back pocket, and the CO and NH of the urea moiety would be expected to interact with the conserved Glu883 and Asp982 of Tie-2.

Compound **7k** was evaluated for its ability to inhibit the growth of human umbilical vein endothelial cells (HUVECs) stimulated by VEGF and the autophosphorylation of c-fms-Tie-2 kinase chimeric receptor transfected in 3T3 cells. Additionally, a cytotoxic cell assay was conducted using the HFF cell line.[11] Data are summarized in Table 3, which show a good correlation between enzyme and cellular potency. Importantly, there is >10-fold selectivity between HUVECs and HFF cell lines suggesting that general cytotoxicity is not the mechanism for inhibition of HUVEC growth.

In conclusion we have discovered 4-NH_2-furo[2,3-*d*]pyrimidines bearing a diarylurea substituent at the 5-position as a novel class of highly potent inhibitors of the angiogenesis receptor type tyrosine kinases, VEGFR2 and Tie-2. In addition to activity in isolated enzyme assays, Compound **7k** exhibits potent cellular inhibitory activity versus proliferation of HUVEC and autophosphorylation of the c-fms-Tie-2 receptor. The arylurea function plays an important role by means of hydrophobic and hydrophilic interactions which have been verified through X-ray analysis.

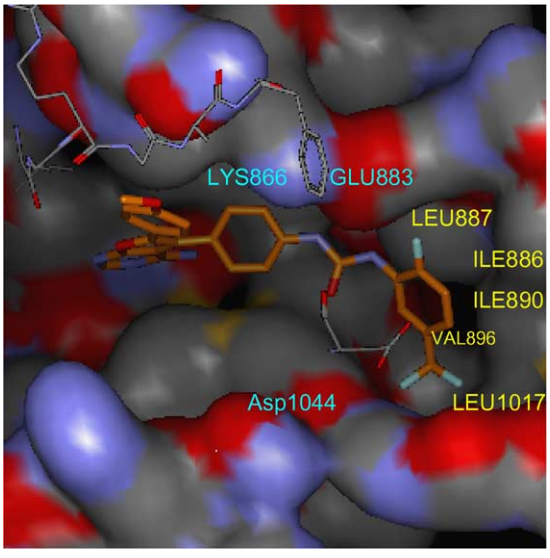


Figure 3. Angle from phosphate binding region. Protein surface was added and colored by atom (C, gray; N, purple; O, red; S, yellow; F, cyan).

Table 3. Cellular inhibitory activity of compound **7k** expressed as IC_{50} value in micromolars

Compound	Tie-2 autophosphorylation (μM)	HUVECv (μM)	HFF (μM)
7k	0.0063	0.045	0.79

Acknowledgements

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89%, (g) 56%, (c) 69%, (d) 88%, (e) 31%, and the reaction with corresponding isocyanate is 60%.

8. The enzyme assay was performed by HTRF (homogeneous time-resolved fluorescence) method using baculovirus-expressed recombinant protein. HTRF is based on the proximity of a donor label (europium chelate) and acceptor label (allophycocyanin, APC) which have been brought together by a specific binding reaction. When the two entities come into close proximity and upon excitation, energy transfer occurs and APC re-emits a specific long-lived fluorescence at 665 nm. The kinases were purified as the intracellular domain of human Tie-2 or VEGFR2 fused by GST tag. The catalytic activity of each kinases was detected by a biotinylated synthetic peptide as a substrate, biontin-C6-LEARLVAYEGWVAGKKK-amide, and biotin-aminohexyl-EEEEYFELVAKKKK-NH_2 for TIE-2 and VEGFR2, respectively. Phosphophorylated substrate is measured by streptavidin linked-APC (Molecular Probes) and europium-labeled anti-phosphorylated tyrosine antibody (Perkin Elmer). Assay conditions are as follows. Tie-2: pre-activated GST-TIE-2 with 2 mM ATP, 5 mM $MgCl_2$ and 12.5 mM DDT was incubated for 30 min with 1 μM peptide, 80 μM ATP, 10 mM $MgCl_2$, 0.1 mg/ml BSA and test compound in 1 mM HEPES. VEGFR2: GST-VEGFR2 was incubated for 40–60 min with 360 nM peptide, 75 μM ATP, 5 mM $MgCl_2$, 0.1 mM DTT, 0.1 mg/ml BSA and test compound in 100 mM HEPES.
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10. The PDB deposition code is 1YWN for this structure.
11. Tie-2-autophosphorylation at the cellular level was measured by ELISA using recombinant mouse 3T3 cells (TIE-2/c-fms) that stably overexpress the chimeric protein of c-fms extracellular domain and Tie-2 intracellular domain. The test compound was incubated with TIE-2/c-fms cells for 1 h followed by the activation of TIE-2-c-fms receptor using c-fms ligand, MCSF (macrophage colony stimulating factor). For capture ELISA with anti-c-fms antibody, phosphorylation was detected using phosphotyrosine antibody and colorimetric substrate.