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Design and effective synthesis of novel templates, 3,7-diphenyl-4-amino-thieno and furo-[3,2-*c*]pyridines as protein kinase inhibitors and in vitro evaluation targeting angiogenetic kinases

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Abstract—A novel class of 3,7-diphenyl-4-amino-thieno and furo[3,2-*c*]pyridine has been designed based on pharmacophore models of ATP competitive kinase inhibitors. Versatile synthetic methods via double Suzuki coupling to explore SAR have been established and potent inhibitors against angiogenetic targets, VEGFR2, Tie-2, and EphB4, have been successfully discovered.

Protein kinases play important roles in cell-signaling pathways regulating a variety of cellular functions. Aberrant behaviors in such signal pathways are linked to a variety of diseases including cancer, diabetes, and inflammation. Receptor tyrosine kinases primarily expressed on endothelial cells mediate cellular functions such as proliferation, survival, and migration, and are deeply involved in angiogenesis, which are linked to tumor metastasis.[1] VEGFR2 and Tie-2 are two examples that have been well studied and are recognized as key players in angiogenesis.[2] Likewise, EphB4 is considered as one of the most attractive targets since its cell signaling is linked to endothelial cell migration, proliferation, and is implicated in regulating cell morphology, attachment, and motility through regulation of integrin-dependent cell adhesion.[3] Recent series of exciting development in clinical candidates targeting angiogenesis, such as anti-VEGF antibody, and small molecules inhibiting VEGFR2 and additional complementary targets such as b-Raf and PDGFR lends support to the anti-angiogenesis approach to deliver new types of anti-cancer drugs for patients.[4]

To date a great number of efforts at discovering small molecules to inhibit the catalytic domain of disease-associated kinases have been expended. To elucidate their inhibitory activity, crystal structures of protein kinases complexed with different classes of inhibitors were obtained. Furthermore, structure-based pharmacophore modeling centered on binding in a catalytic domain has emerged,[5] and as a result of such knowledge, a number of ATP competitive inhibitors have been successfully reported.

The present communication describes a knowledge-based approach to the design of two novel templates, 3,7-diaryl-4-amino-thieno and furo[3,2-*c*]pyridine, based on the pharmacophore model for ATP competitive kinase inhibitors, and the subsequent synthesis leading to a series of potent EphB4 inhibitors. We also wish to report additional studies based on the SAR of the VEGFR2 and Tie-2 dual inhibitors 4-amino-furo[2,3-*d*]pyrimidines previously reported,[6] resulting in highly potent triple inhibitors of VEGFR2, Tie-2, and EphB4 with potential as anti-angiogenic molecules.

In a previous paper, we reported on a series of novel 4-amino-furo[2,3-*d*]pyrimidines as VEGFR2 and Tie-2 dual inhibitors.[6] X-ray analysis of their complex with VEGFR2 provided the information that the NH_2 and nitrogen of the aminopyrimidine moiety form hydrogen bond interaction with the hinge region, which is usually observed for ATP competitive inhibitors. According to a widely accepted pharmacophore model for the design of

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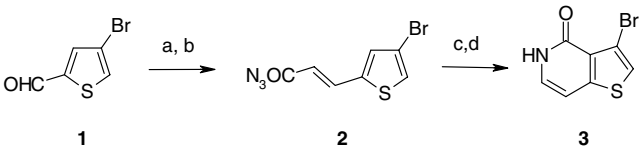
ATP competitive kinase inhibitors, N1 could be replaced by a different atom and substitutions at that position can extend to the outer hydrophobic pocket (Fig. 1) which allow for additional inhibitor-enzyme affinity and the opportunity to exploit residue differences for inhibitory selectivity. Based on such findings and hypotheses, thieno and furo-3,7-diaryl-4-amino-[3,2-*c*]pyridine were designed as core scaffolds.

The chemistry for generating a diverse array of compounds was accomplished in a versatile way using procedures illustrated in Schemes 2 and 3.[7] In the procedure to derivatize the 3-position, selective double Suzuki-coupling via differentiated halides afforded a set of derivatives **6** with diversity at 3-position (Scheme 2). In another procedure to derivatize the 7-position, sequential halogenations and Suzuki-coupling provided derivatives **9** with diverse 7-substituents (Scheme 3). The core structure was synthesized using the following methods.[8] Commercial starting material 4-bromo-thiophene-carbaldehyde **1** was converted to the acrylic acid by condensation with malonic acid, which was transformed to azide **2**. After a Curtius-type rearrangement and sequential isomerization to the E form with a catalytic amount of iodine, cyclization took place under thermal conditions to give thieno[3,2-*c*]pyridin-4-one core **3** (Scheme 1). Iodination of **3** with NIS occurred at the 7-position, and this was followed by chlorination and amination to give key intermediate **4**. Chlorination of **3** with phosphorus oxychloride and amination with ammonia solution provided another intermediate **7**.

To study the effect of backpocket hydrophobic interaction on inhibitory activity, the aryl moiety at the 3-position was diversified. Here, the 3,4,5-trimethoxyphenyl group, which is known to be well tolerated at the solvent front of the active site from our previous kinase system-based research work, was selected as a substituent kept constant at the 7-position.[9] The inhibitory activities against the EphB4 enzyme for the resultant 4-amino-3-aryl-7-(3,4,5-trimethoxylphenyl)-thieno[3,2,*c*]pyridines **6** are summarized in Table 1.[10]

As the data indicate, compounds with hydrophobic chloro groups regardless of substitution position (**6d**, **6e**, and **6j**) show submicromolar inhibitory activity. However, those with somewhat hydrophilic substituents



Scheme 1. Synthesis of core structure, thieno[3,2-*c*]pyridin-4-one. Reagents and conditions: (a) malonic acid, piperidine, pyridine, 100 °C, quant.; (b) DPPA, Et_3N, THF, rt, 75%; (c) toluene, 120 °C; (d) cat. I_2, *o*-dichlorobenzene, 170 °C, 48%.

such as 3-acetamido, 4-acetyl, and 4-methylsulfonyl groups (**6f**, **6g**, and **6i**) are relatively inactive. Other substituents such as 4-methoxyphenyl, naphthyl, and pyridyl provided analogues with moderate potency (**6b**, **6c**, and **6h**). The need for a phenyl group for inhibitory activity is evidenced by the lack of activity of **6a**.

We next turned our attention to exploration of SAR at the 7-position utilizing the procedure illustrated in Scheme 3. 3-Chloro-4-fluoro-phenyl was selected as a fixed substituent at the 3-position based on high potency of such a substituent, as shown in Table 1. The enzyme data, as well as the data from the cell assay, are summarized in Table 2. The cell assay measured the inhibition of autophosphorylation of the chimeric c-fms-EphB4 receptor transfected in 3T3 cell.[11] In the current SAR studies, analogues with hydrophilic substituents (**9c**, **9d**, **9e**, and **9f**) enhanced enzyme potency by 11-fold compared to that of **6e**.[12] Here, the lack of enzyme activity for the unsubstituted **9a** and the bromo intermediate **9b** suggests the need for the aromatic ring positioned in solvent front region. These data also support the pharmacophore model mentioned above for the design of ATP competitive kinase inhibitors, which also suggests that the presence of the hydrophobic residues in the solvent front region can be exploited to increase binding affinity for the inhibitors. These compounds have the added advantage of exhibiting nanomolar cellular inhibitory activity of autophosphorylation of the kinase domain.

We next utilized the SAR that was developed for the 4-amino-fluoro[2,3-*d*]pyrimidines, reported previously as VEGFR2 and Tie-2 dual inhibitors,[6] wherein 2-fluoro-5-trifluoromethyl-phenylurea moiety attached at the 3-phenyl moiety was found to enhance dramatically both

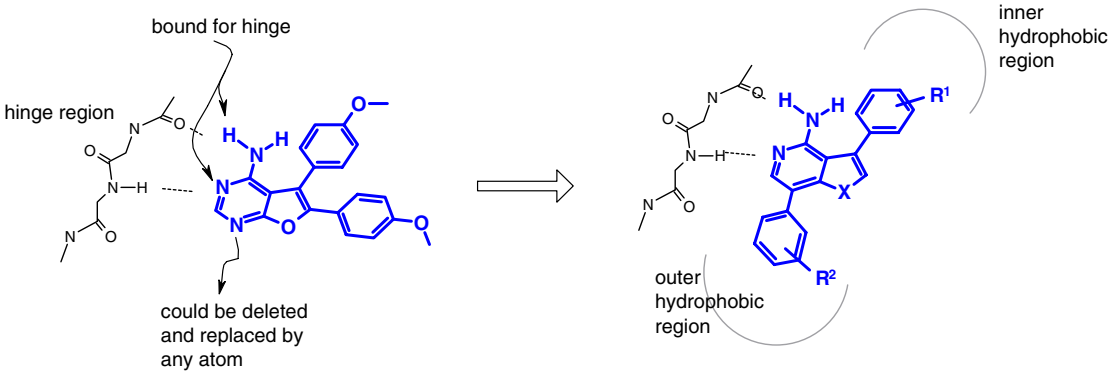
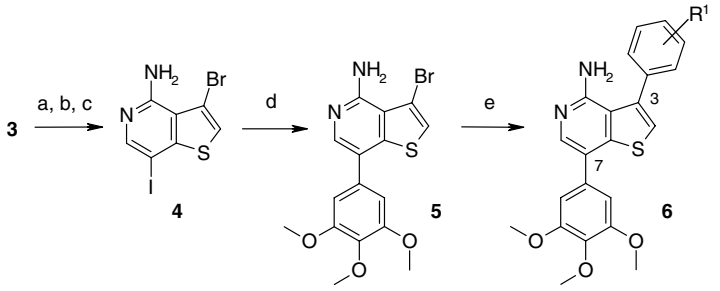
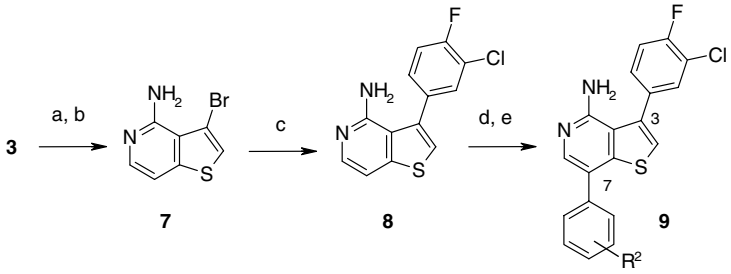


Figure 1. Design of thieno and furo-3,7-diaryl-4-amino-[3,2-*c*]pyridine.

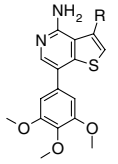


Scheme 2. Reagents and conditions for derivatization on 3-position: (a) NIS, THF-DMF, 40 °C, 66%; (b) $POCl_3$, 120 °C, quant.; (c) 28% ammonia solution, dioxane in a sealed tube, 150 °C, 50%; (d) 3,4,5-trimethoxybenzeneboronic acid, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C, 53%; (e) aryl boronic acid/ester, $Pd(PPh_3)_4$, 2 M Na_2CO, DME, 80 °C, 17–50%.



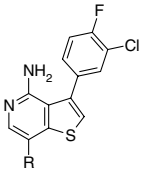
Scheme 3. Reagents and conditions for 7-position: (a) $POCl_3$, 120 °C, quant.; (b) 28% ammonia solution, dioxane in a sealed tube, 150 °C, 57%; (c) 3-chloro-4-fluoro-benzeneboronic acid, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C, 91%; (d) NBS, THF, 0 °C, quant.; (e) aryl boronic acid/ester, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C, 35–65%.

Table 1. EphB4 kinase enzyme inhibition of 4-amino-3-aryl-7-(3,4,5-trimethoxylphenyl)-thieno[3,2,*c*]pyridines **6**



Compound	R	EphB4 IC_{50} (nM)
6a	Br	>25,000
6b	4-OMe-phenyl	7100
6c	β-Naphthyl	2200
6d	4-Cl-phenyl	190
6e	3-Cl-4-*F*-phenyl	110
6f	3-acetamide-phenyl	>25,000
6g	4-Acetyl-phenyl	>25,000
6h	3-Pyridyl	3900
6i	4-Methylsulfonyl-phenyl	>23,000
6j	2,3-Dichloro-phenyl	230

Table 2. EphB4 kinase enzyme and cellular inhibitory activity of 4-amino-5-(3-chloro-4-fluoro-phenyl)-7-aryl-thieno[3,2,*c*]pyridines **9**



Compound	R	EphB4 IC_{50} (nM)	Cfms-EphB4 (nM)
9a	H	1350	ND
9b	Br	575	ND
9c	3-Acetamide-phenyl	44	20
9d	3-Pyridyl	51	29
9e	3-Methylsulfonyl-phenyl	59	18
9f	3-Sufonylamide-phenyl	21	19

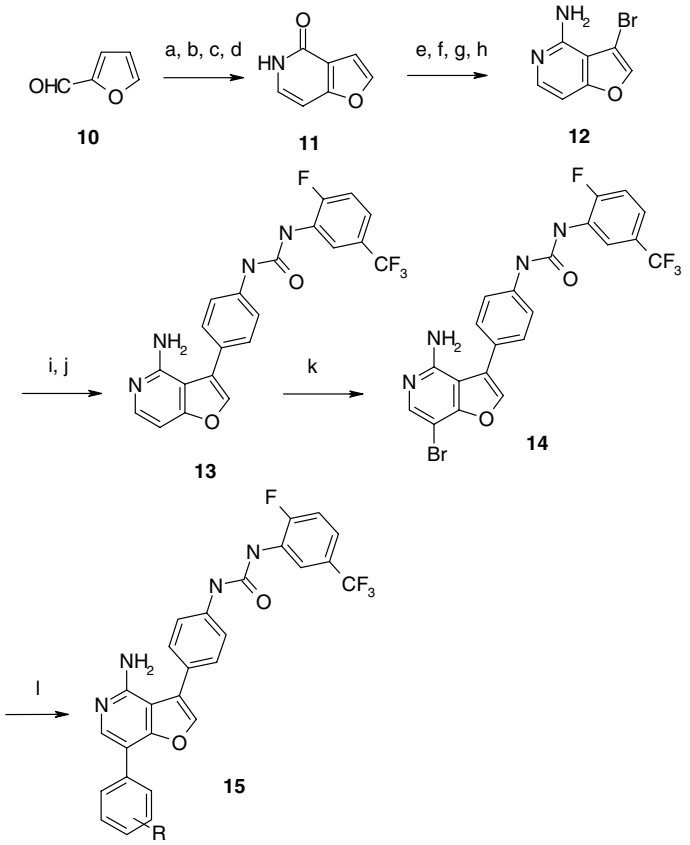
Table 3. Enzyme inhibitory activity against angiogenesis related targets of 4-amino-7-aryl-furo[3,2-*c*]pyridines **15**

Compound	R	VEGFR2[a] (nM)	Tie-2[a] (nM)	EphB4[a] (nM)
15a	H	30	46	14
15b	3-Chlorophenyl	933	2188	389
15c	3-Sulfonylamide phenyl	4.2	17	0.4

[a] IC_{50} values are means of greater than two experiments.

VEGFR2 and Tie-2 enzyme potency. This work, as summarized in Table 3, resulted in compounds manifesting enzyme inhibitory activity versus VEGFR2, Tie-2, and EphB4.[13] The chemistry is illustrated in Scheme 4.[14]

Compared with null derivative **15a**, 3-Cl derivative **15b** led to lower potency, while 3-sulfonamide, **15c**, showed stronger inhibitory activity against VEGFR2, Tie-2, and EphB4.[12] The environment, where substituents on the phenyl ring at 7-position could be exposed, is composed of Ala and Asp, Asn and Asn for EphB4, Tie-2, and VEGFR2, respectively. Thus hydrophilic interactions at this position are expected to affect enzyme potency. Additionally 2-fluoro-5-trifluoromethyl-phenylurea moiety is expected to interact and bind tightly in the hydrophobic backpocket resulting in highly potent



Scheme 4. Synthesis of 4-amino-7-aryl-furo[3,2-*c*]pyridine. Reagents and conditions: (a) malonic acid, piperidine, pyridine, 100 °C, 78%; (b) DPPA, Et_3N, THF, rt, 68%; (c) toluene, 120 °C; (d) cat. I_2, *o*-dichlorobenzene, 170 °C, 63% for step c and d; (e) $POCl_3$, 120 °C, 84%; (f) Br_2, CCl_4, rt; (g) DBU, THF, rt, 85% for step f and g; (h) 28% NH_3 solution, dioxane in a sealed tube, 150 °C, 47%; (i) 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C, 83%; (j) 2-fluoro-5-(trifluoromethyl)phenyl isocyanate, THF, 80%; (k) NBS, THF, −78 °C to 0 °C, 41%; (l) aryl boronic acid/ester, $Pd(PPh_3)_4$, 2 M Na_2CO_3, DME, 80 °C, 31% and 34% for **15b** and **15c**, respectively.

inhibitors, similar to that shown by the 4-amino-furo[2,3-*d*]pyrimidines.

Compound **9f** was docked into a homology model of EphB4 to understand its potency, as shown in Figure 2. The NH_2 and nitrogen of the aminopyridine interact with Glu694 and MET696, respectively. 3-Chloro-4-fluoro-phenyl at 3-position is accommodated into the hydrophobic backpocket, additionally sulfonamide is directed toward the solvent front and interacts with either backbone or hydrophilic residues.

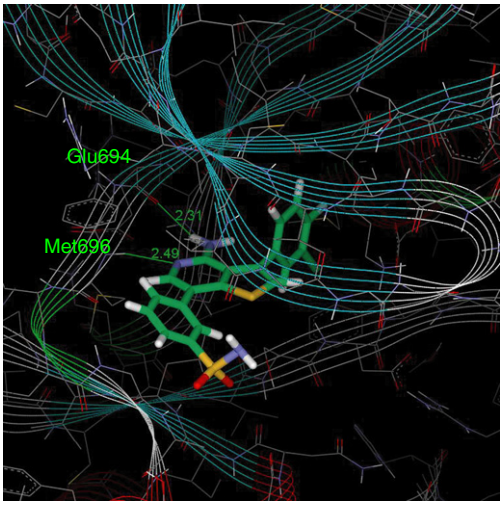


Figure 2. Docking compound **9f** into EphB4 homology model.

In conclusion, we had established a versatile and practical route to the 3,7-diaryl-4-amino-thieno[3,2,*c*]pyridine to optimize their kinase inhibitory activities and discovered potent EphB4 inhibitor **9f** at both enzyme and cellular levels. Additionally application of the chemistry to furo-derivatives had afforded very strong inhibitor, **15c**, against angiogenesis-related targets.

Acknowledgments

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10. For Tie-2 and VEGFR2, the enzyme assay was performed by the 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 emits a specific long-lived fluorescence at 665 nm. For EphB4, the enzyme assay used the scintillation proximity method where the localization of a radiolabeled phosphate near scintillant-containing beads generates a signal. The kinases were purified as the intracellular domain of human Tie-2, VEGFR2, or EphB4 fused by GST and/or 6×His tags. In the case of VEGFR2, the enzyme contains both GST and 6×His tags. The catalytic activity of each kinase was detected by using a biotinylated synthetic peptide as a substrate, biotin-C6-LEA-RLVAYEGWVAGKKK-amide, biotin-aminohexyl-EEEEYFELVAKKKK-NH2, and biotin-aminohexyl-MAHFENYEFFHAKKK-NH2 for Tie-2, VEGFR2, and EphB4, respectively. Phosphorylated substrate is measured by streptavidin-linked-APC (Molecular Probes) and europium-labeled anti-phosphorylated tyrosine antibody (Perkin-Elmer) or by streptavidin-coated SPA beads (Amersham-Pharmacia). Assay conditions are as follows. Tie-2: GST-Tie-2 was preactivated with 800 μM ATP, 1 mM DTT, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 0.01% Tween 20 in 100 mM HEPES, pH 7.5, for 1–2 h. The preactivated enzyme was then incubated for 1–3 h with 1 μM peptide, 20 μM ATP, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL BSA, 0.01% Tween 20, and test compound in 100 mM HEPES, pH 7.4. VEGFR2: GST-6×His-VEGFR2 was preactivated with 100 μM ATP, 0.3 mM DTT, 0.1 mg/mL BSA, and 10 mM $MgCl_2$ in 100 mM HEPES, pH 7.5, for 20 min. The preactivated enzyme was then incubated for 90 min with 360 nM peptide, 50 μM ATP, 10 mM $MgCl_2$, 0.3 mM DTT, 0.1 mg/ml BSA, and test compound in 100 mM HEPES, pH 7.5. EphB4: GST-EphB4 was preactivated with 50 μM ATP and 10 mM $MgCl_2$ and was then incubated for 3 h with 8 μM peptide, 1 μM ATP, 5 μCi/mL 33P-ATP, 10 mM $MgCl_2$, 1 mM DTT, 5 mM KCl, 1 mM CHAPS, 0.1 mg/mL BSA, and test compound in 100 mM HEPES.
11. EphB4 autophosphorylation at the cellular level was determined by ELISA using recombinant NIH3T3 cells (cFms-EphB4) with the stable expression of the chimeric receptors of cFms-derived extracellular domain and EphB4-derived intracellular domain. The test compound was incubated with cFms-EphB4 for 1 h after serum starvation. The cells were stimulated with a specific ligand, M-CSF (macrophage colony stimulating factor), to induce the activation of the cFms-EphB4 receptors. The phosphorylation level of the receptors was measured by the incubation of the ELISA plate capturing the protein via anti-cFms antibody with anti-phosphotyrosine antibody and visualized by incubating with chemiluminescent substrates.
12. Compounds **9f** and **15c** showed relatively weak inhibitory activity (IC_{50} > 10 μM) against following targets. Akt3, CDK2, ErbB2, GSK3b, IKK2, MAPKAPK2, and PLK1. IC_{50} of **9f** against VEGFR2 and Tie-2 is 1.9 μM and over 20 μM, respectively.
13. Compound **15c** showed comparable potency against VEGFR2 and Tie-2 against 4-amino-3-(4-((2-fluoro-5-(trifluoromethyl)phenyl)amino-carbonylamino)phenyl)-2-(4-methoxyphenyl)furo[2,3-*d*]pyrimidine, which was published in our previous report (see Ref. 6). It should be noted that assay condition was slightly modified from the previous one.
14. Bromination of compound **13** occurred at 2-position as well as 7-position, resulting in low yield.