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Orally active 4-amino-5-diarylurea-furo[2,3-*d*]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2

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Abstract—During our effort to develop dual VEGFR2 and Tie-2 inhibitors as anti-angiogenic agents for cancer therapy, we discovered 4-amino-5-(4-((2-fluoro-5-(trifluoromethyl)phenyl)- aminocarbonylamino)phenyl)furo[2,3-*d*]pyrimidine (**8a**) possessing strong inhibitory activity at both the enzyme and cellular level against VEGFR2 and Tie-2. Compound **8a** demonstrated high pharmacokinetic exposure through oral administration, and showed marked tumor growth inhibition and anti-angiogenic activity in mouse HT-29 xenograft model via once-daily oral administration.

Angiogenesis, the formation of new blood vessels from existing vasculature, has been considered a critical event for the growth and metastasis of solid tumors since Folkman first proposed the hypothesis that tumors cannot grow beyond 2–3 mm in the absence of new vasculature.[1] Numerous studies have shown that angiogenic growth factors and cellular kinase receptors regulate a variety of cellular events relating to angiogenic processes. Two growth factor families, VEGFs and Angiopoetins, are of particular interest since their receptors are expressed primarily on endothelial cells and play a direct role in such angiogenic processes.[2] The VEGFs are required for vasculogenesis and angiogenic sprouting, and act through receptor tyrosine kinases (VEGFR-1, -2, and -3). VEGFR2 mediates endothelial cell proliferation, differentiation, and microvascular permeability.[3] The Angiopoetins (Ang-1 and Ang-2), on the other hand, have been implicated in the further remodeling of initial microvasculature and exert biological actions through the Tie-2 receptor.[4]

It is now well established that inhibition of VEGFR2 leads to suppression of angiogenesis and tumor growth. Interference of VEGFR2 signaling with a truncated VEGFR2 gene lacking the kinase domain or inhibition by small molecule inhibitors was shown to significantly suppress the growth of a variety of solid tumors.[5,6] Similarly, suppression of Tie-2 pathways was also shown to have anti-angiogenic and anti-tumor activities. Antisense reagents of Ang-1 RNA could effectively reduce Ang-1 production and inhibit angiogenesis and tumor growth.[7] Furthermore, it was shown that interfering with either the VEGFR2 or the Tie-2 pathways led to inhibition of human melanoma xenograft growth.[8]

Based on this body of evidence, we focused on developing small molecules with dual inhibitory activity against both VEGFR2 and Tie-2, expecting the simultaneous inhibition of both enzymes would show synergistic effects through affecting critical stages of blood vessel formation, and thus leading potentially to a new approach to cancer therapy.

From our initial high throughput screen, 4-amino-5,6-bis[(4-methoxy)phenyl]furo[2,3-*d*]pyrimidine **1** as identified as a dual inhibitor with moderate potency against both the VEGFR2 and Tie-2 enzymes. Subsequent

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modification successfully led to 4-amino-5-diarylurea-6-aryl-furo[2,3-*d*]pyrimidines such as compound **2** that exhibits high potency in both VEGFR2 and Tie-2 enzyme and cellular assays (Fig. 1).[9] In this paper, we describe our further efforts to develop a candidate quality molecule within the 4-amino-5-diarylurea-furo[2,3-*d*]pyrimidine series, possessing both VEGFR2/Tie-2 enzyme and cell dual inhibitory activity, as well as multiple kinase inhibitory profiles affecting angiogenesis. In addition, we report the oral pharmacokinetic exposure and in vivo efficacy of a representative analogue **8a**.

The VEGFR2 X-ray crystal structure of the potent VEGFR2 and Tie-2 dual inhibitor 4-amino-furo[2,3-*d*]pyrimidine **2** showed that among the key interactions the diaryl urea moiety serves as a hook extended into the hydrophobic back pocket of the kinase domain and the aminopyrimidine as an anchor bound to the hinge region. This serves as a basis for the rationale behind the potency observed. Superimposing **2** onto the equally potent dual VEGFR2/Tie-2 inhibitor 5-diarylurea-oxy-benzimidazole derivative **3** on the basis of similar binding motif in the X-ray crystal structure suggested that deleting the 4-methoxyphenyl group at the 6-position of **2** might not affect its potency (Fig. 2).[10] Since such a deletion lowers the molecular weight, this may potentially lead to compounds that show better PK exposure. Moreover, other functional groups can be introduced into the 6-position to fine-tune physicochemical properties such as solubility and lipophilicity, if the need arises. To validate this hypothesis, the 6-position-depleted 4-amino-5-diarylurea-furo[2,3-*d*]pyrimidines were synthesized.

The synthesis is depicted in Scheme 1. 4-Amino-acetophenone **4** was converted to 2-hydroxyacetophenone **5** by bromination and hydroxylation with potassium formate. Subsequent reaction with malononitrile in the presence of diethylamine led to furan **6**, which was treated with triethyl orthoformate, followed by amination and cyclization with sodium ethoxide, providing 4-amino-5-(4-acetamidophenyl)-furo[2,3-*d*]pyrimidine **7**. After removal of the acetyl group, **7** was treated with various isocyanates to provide the corresponding urea derivatives **8**.[11]

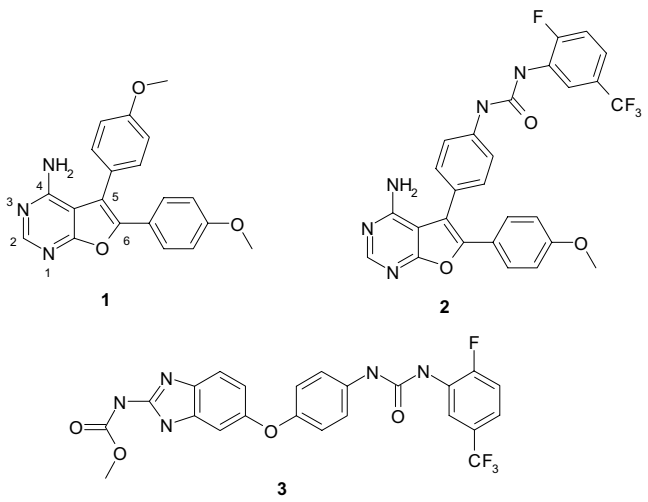


Figure 1. Structure of 4-amino-5,6-diaryl-furo[2,3-*d*]pyrimidine **1**, **2**, and 5-diarylurea-oxy-benzimidazole **3**.

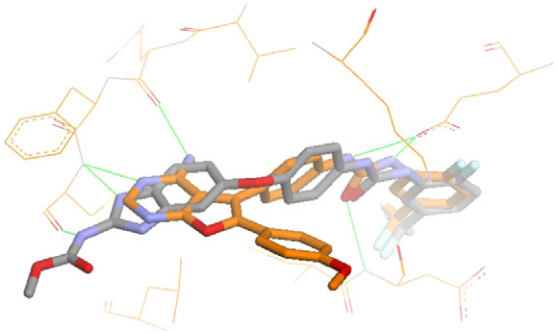
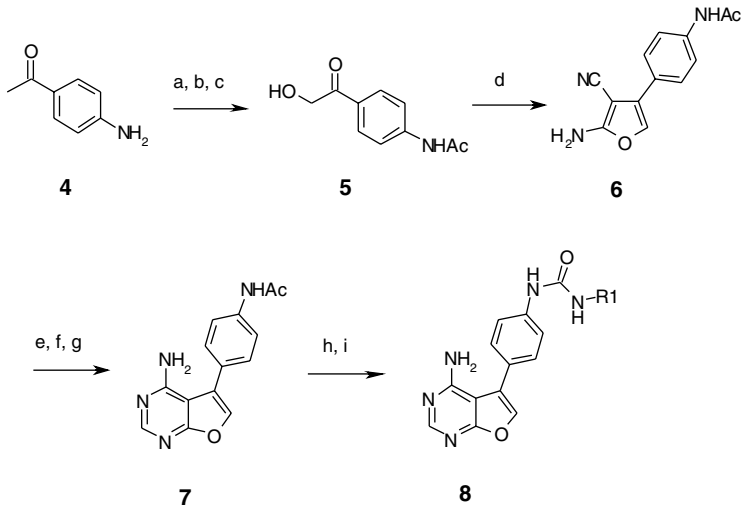


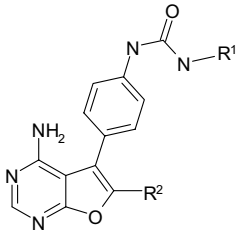
Figure 2. Overlay of 5-diarylurea-oxy-benzimidazole into 4-amino-5-diarylurea-6-aryl-furo[2,3-*d*]pyrimidine complexed with VEGFR2. 5-diarylurea-oxy-benzimidazole 3 (gray) was superimposed into X-ray structure of 4-amino-5-diarylurea-6-aryl-furo[2,3-*d*]pyrimidine 2 complexed (orange) with VEGFR2. Key interactions between inhibitor and protein are shown by green lines.

pIC_{50} values for VEGFR2 and Tie-2 inhibitory activity were determined by HTRF (homogeneous time-resolved fluorescence) method.[12] The catalytic activity of kinases was measured by phosphorylated biotin-peptide conjugate by means of streptavidin linked-APC and europium-labeled anti-phosphotyrosine antibody. As shown in Table 1, deletion of the aryl ring at the 6-position completely retains the enzyme activity comparable to that of the 6-methoxyphenyl analogue **2**. In the preliminary SAR studies of the 4-amino-5,6-diaryl-furo[2,3-*d*]pyrimidine series, both aromatic substituents at 5- and 6-position were deemed important for inhibitory activity;[9] however, the diarylurea moiety now picks up potency either with or without aromatic substituents at 6-position as suggested from the studies derived from the superimposition with 5-diarylurea-oxy-benzimidazole series. This result suggested that the paramount interactions in the current series are those of the diarylurea with the hydrophobic back pocket and the proton donor/acceptor interactions in the hinge region between the enzymes and the inhibitors.[13] In Table 1, the SAR of the terminal substitution of the urea portion (R^1) for inhibitory activity against VEGFR2 and Tie-2 is summarized. As shown in the table, 3-trifluoromethylphenyl is important for potency against both VEGFR2 and Tie-2 (compound **8d**). The 17- and 42-fold reduction in potency against Tie-2 and VEGFR2, respectively, of the methoxy analogue **8f** compared to that of **8a** clearly indicated the importance of the hydrophobic interaction between the inhibitor and the hydrophobic amino acid residues of the back pocket. Bulky substituents such as 3-phenoxyphenyl (**8c**) reduced activity against both enzymes, especially for VEGFR2. Introduction of a chloro substituent instead of the fluoro group of **8a** decreases activity against both Tie-2 and VEGFR2 by 16- and 23-fold, respectively (**8e**). Similar reduction in activity was observed by the bulky naphthyl analogues **8g** and 3,5-ditrifluoromethylphenyl **8b**. 2-Naphthyl analogue **8h** increased the potency slightly compared with **8g**,



Scheme 1. Reagents and conditions: (a) Ac_2O, toluene, rt, 96%; (b) Br_2, AcOH, 60 °C, 63%; (c) potassium formate, $NaHCO_3$, EtOH–H_2O, 40 °C, 50%; (d) malononitrile, Et_2NH, DMF, rt, 81%; (e) $HC(OEt)_3$, Ac_2O, 100 °C; (f) NH_3, EtOH–THF, rt; (g) NaOEt, EtOH–THF, rt, 54% for three steps; (h) 2 M KOH, EtOH–H_2O, 60 °C, 75%; (i) isocyanates, THF, rt, 95% for 2-fluoro-5-(trifluoromethyl)phenyl isocyanate.

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



Compound	R^1	R^2	Tie-2 pIC_{50}	Tie-2 SD	VEGFR2 pIC_{50}	VEGFR2 SD
2	2-F-5-CF_3-Ph	4-OMe-Ph	ND (8.61)[b]	ND	7.81 (8.56)[b]	$n = 1$
8a	2-F-5-CF_3-Ph	H	7.87 (8.62)[b]	0.29, $n = 4$	8.11 (8.54)[b]	0.25, $n = 6$
8b	3,5-Di- CF_3-Ph	H	6.42	$n = 1$	6.86	$n = 1$
8c	3-Phenoxy-Ph	H	6.39	$n = 1$	5.76	$n = 1$
8d	3- CF_3-Ph	H	7.99	$n = 1$	8.15	$n = 1$
8e	2-Cl-5- CF_3-Ph	H	6.69	0.11, $n = 3$	6.73	0.15, $n = 5$
8f	2-F-5-OMe-Ph	H	6.65	$n = 1$	6.48	0.59, $n = 2$
8g	1-Naphthyl	H	6.74	$n = 1$	6.35	$n = 1$
8h	2-Naphthyl	H	7.09	$n = 1$	7.62	$n = 1$

[a] Number of concentration points per curve for pIC_{50} determination is 11.
[b] The value in the parentheses indicates pIC_{50} value in a previous assay format.[9]

indicating some space for meta- and para-direction of the terminal phenyl moiety.

Compound **8a** was evaluated for cellular activity based on the growth of human umbilical vein endothelial cells (HUVECs) stimulated by VEGF or the autophosphorylation of c-fms-Tie-2 chimeric receptor transfected into 3T3 cells.[9] Additionally, cytotoxic assays were conducted using the HFF and HT-29 cell lines.[14] As indicated in Table 2, compound **8a** exhibited potent cellular inhibitory activity against the growth of HUVECs and autophosphorylation of c-fms-Tie-2 chimeric receptor. In addition, there was over 900-fold selectivity between inhibition of growth of HUVECs and HFF cell lines,

Table 2. Cellular inhibitory activity of compound **8a** expressed as IC_{50}

Cellular inhibitory activity, IC_{50} (nM)			
HUVECv	Tie-2 Autophosphorylation	HFF	HT-29
2.6	18.3	2506	5084

IC_{50} values are averages from more than two separate experiments.

Table 3. Systemic exposure via oral administration in the male CD-1 mouse of compound **8a**

Systemic exposure in mice (10.7 mg/kg, po)			
C_{max} (ng/mL)	T_{max}[a] (min)	AUC0-t (min μg/mL)	DNAUC0-t (min kg/L)
2724 ± 588	480, 120, 120, 120	1201 ± 272	112 ± 22

Abbreviations: C_{max}, maximum blood concentration attained after oral gavage; T_{max}, time C_{max} was attained; AUC0-t, area under the blood concentration–time curve truncated to the last time point with measurable concentrations; DNAUC0-t, dose-normalized AUC.
[a] Individual values shown due to variability.

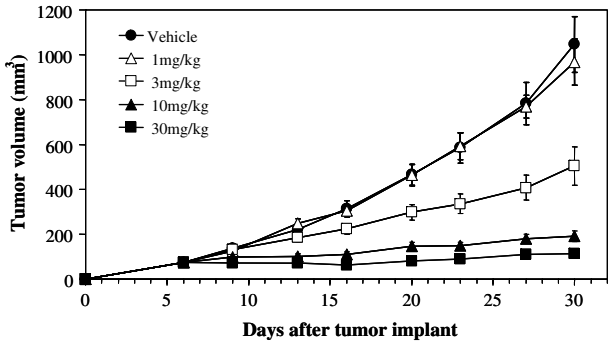


Figure 3. Effect of **8a** or vehicle on HT-29 tumor growth in xenograft model. HT-29 human adenocarcinoma cells (2×10^6 cells) were implanted sc in the flank of female CD-1 nude mice. Five days after the implantation, once-daily oral treatment with **8a** or vehicle (1% CMC-Na/saline) was started and continued for the experimental period. Data points represent a means ± SE ($n = 8$).

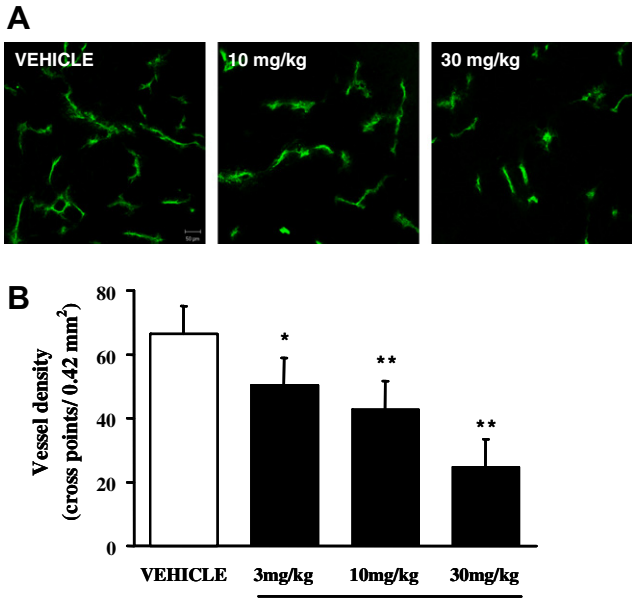


Figure 4. Effect of **8a** on vascular density in HT-29 tumor xenografts. A view of the square field (686-μm width) on frozen section was scanned with a confocal laser-scanning microscope (A), and the vessel density (B) in 'angiogenic hot spots' was determined by image analysis according to the method described by Rieder et al.[14]. The column with bar represents a means ± SE ($n = 4$). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, as compared to VEHICLE group (Dunett's multiple comparison test).

suggesting that general cytotoxicity is not the mechanism for inhibition of HUVEC growth. Preliminary pharmacokinetic studies in mouse at a dose of approximately 10 mg/kg of compound **8a** formulated with CMC-Na/saline showed excellent exposure after oral administration (Table 3).[15]

In vivo anti-tumor activity of compound **8a** was evaluated in a mouse HT-29 tumor xenograft model. HT-29 human colon adenocarcinoma cells were cultured and implanted into nude mice and 5 or 6 days later tumor size and volume were measured. Compound **8a** was suspended in 1% carboxymethylcellulose sodium salt saline solution (1% CMC-Na/saline) and orally administered at doses of 1, 3, 10, and 30 mg/kg once daily. For the control, 1% CMC-Na/saline was given. Through the experimental period, the tumor volume and the body weight were monitored. As shown in Figure 3, oral administration of compound **8a** at 1–30 mg/kg dose-dependently inhibited HT-29 tumor growth. At 30 mg/kg tumor growth was almost completely suppressed, although the body weight loss was observed during the late stage of the experimental period. Nevertheless, at doses of 3 and 10 mg/kg the tumor growth was significantly inhibited without body weight loss.

Anti-angiogenic activity of compound **8a** was also examined in mouse HT-29 tumor xenograft model at doses of 3, 10, and 30 mg/kg or vehicle (1% CMC-Na/saline) for 6 days. Vascular endothelial cells in HT-29 xenografts were labeled with FITC-lectin and blood vessels in the section of 'angiogenic hot spots', which are the most densely vascularized areas of the tumor, were detected as fluorescent images by confocal laser-scanning microscopy. Blood vessel images were digitized and quantified according to the method described by Rieder et al.[16] in which the vascular density was determined by the number of the cross points between digitized vessel images and computer-generated grid (40 μm-interval). Once-daily oral treatment of HT-29 tumor bearing mice with **8a** decreased blood vessels (Fig. 4A), and anti-angiogenic activity of **8a** was dose-dependent between 3 and 30 mg/kg (Fig. 4B). Vessel density in the tumor section was significantly inhibited 24.3%, 35.6%, and 62.9% by 3, 10, and 30 mg/kg of **8a**, respectively. In addition, the growth of the xenografted tumors was also inhibited during the 6 days of treatment.

Recently, compounds with multiplexed kinase inhibitory profiles have demonstrated efficacy in clinical trials.[17] In fact, recent FDA approval of sorafenib (Nexavar®) which inhibits VEGFR2 plus complementary and additional targets, such as b-RAF and PDGF, would support pursuing a multiplexed kinase inhibitory profile strategy. Compound **8a** was evaluated in a kinase screening panel and demonstrated inhibitory activity against multiple kinases such as VEGFR1, VEGFR3, and PDGFR, which are important kinases required

Table 4. Kinase panel selectivity profile of compound **8a**. Potency is represented by pIC_{50} value[a]

Akt3	CDK2	EGFR	GSK3	SRC	c-FMS	PDGFR	VEGFR1	VEGFR3
4.76 (0.62, $n = 2$)	4.75 (0.31, $n = 7$)	5.0 (0, $n = 2$)	5.66 (0.1, $n = 2$)	6.27 (0.45, $n = 6$)	7.41 (0.32, $n = 2$)	7.56 (0.39, $n = 5$)	8.07 (0.42, $n = 2$)	8.12 (0.20, $n = 2$)

[a] Standard deviation and n values are given in parentheses (n, replicates).

for angiogenesis. The profile data of compound **8a** are shown in Table 4.[18]

In conclusion, as a part of our research for anti-angiogenic agents for cancer therapy, we synthesized 4-amino-furo[2,3-*d*]pyrimidine derivatives, and evaluated their activities as VEGFR2 and Tie-2 kinase inhibitors. Compound **8a** exhibited not only potency in both enzyme and cellular assays, but also exhibited anti-angiogenic and anti-tumor activity in HT-29 tumor xenograft models. These results indicate that compounds of furopyrimidines bearing diarylurea moieties potentially possess promising features as drug candidates for anti-angiogenic cancer therapy.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.077.

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11. Analytical data of compound **8a**: ^{1}H NMR (400 MHz, DMSO-d_6) ppm 7.39–7.42 (m, 1H), 7.47 (d, J = 8.6 Hz, 2H), 7.49–7.54 (m, 1H), 7.63 (d, J = 8.8 Hz, 2H), 7.95 (s, 1H), 8.26 (s, 1H), 8.63 (d, J = 7.1 Hz, 1H), 8.99 (s, 1H), 9.39 (s, 1H). LC/MS: m/z 432 $(M + 1)^+$, 430 $(M - 1)^-$.
12. 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. The kinases were purified as the intracellular domain of human Tie-2 or VEGFR2 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-LEARLVAYEGWVAGKKK-amide, and biotin-aminohexyl-EEEEYFELVAKKKK-NH2, for Tie-2 and VEGFR2, respectively. Phosphorylated 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: 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.
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14. HFF or HT-29 cells (ATCC) were cultured in DMEM, 10% FBS, high glucose, and L-glutamine. Logarithmically growing cells were seeded into 96-well plates (Nunclon polystyrene white part No. 136102) at 500 cells per well and allowed to adhere overnight at 37 °C in a humidified 5% CO_2 incubator. Compounds were dissolved in 100% DMSO and serially diluted twofold for 20 points. Compounds were diluted into DMEM, then diluted into the cell plates at a final dilution of 1:500 yielding a final DMSO concentration of 0.2%. Plates were incubated for three days and then developed with Promega CellTiter-Glo reagent and read on a Perkin-Elmer Victor V plate reader. Curves are fit to a four parameter model using XLfit and the inflection point is recorded as the EC50.
15. In another preliminary PK experiment, compound **8a** showed better exposure compared with compound **2** on C_{max} and AUC (limited) by 1.9- and 2.6-fold, respectively.
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18. Davis, S. P.; Reddy, H.; Caivono, M.; Cohen, P. *Biochem. J.* **2000**, *351*, 95, Compound **2** generally showed similar selectivity profile with compound **8a** (IC_{50} are >10 μM against CDK2, EGFR, GSK3 and <100 nM against SRC and VEGFR3) as far as the inhibitory activity was measured. However, it was found that some substituents on 6-position could change selectivity profile on certain targets (data are not shown).