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N-(Aryl)-4-(azolylethyl)thiazole-5-carboxamides: Novel potent inhibitors of VEGF receptors I and II

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Abstract—Novel potent derivatives of *N*-(aryl)-4-(azolylethyl)thiazole-5-carboxamides are described as inhibitors of vascular endothelial growth factor receptor II (VEGFR-2). Several compounds display VEGFR-2 inhibitory activity reaching $IC_{50} < 100$ nM in both enzymatic and cellular assays. The compounds also inhibit the related tyrosine kinase, VEGFR-1. By controlling the substitution pattern on the 5-carboxamido pharmacophore, both dual and specific VEGFR-2 thiazoles were identified.
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Vascular endothelial growth factors (VEGFs) and their respective family of receptor tyrosine kinases (VEGFRs) are key proteins modulating angiogenesis, the formation of new vasculature from an existing vascular network.[1] Potent, specific, and non-toxic inhibitors of angiogenesis are powerful clinical tools in oncology and ophthalmology.[2,3] Several groups in industry have developed methods for sequestering VEGF. This leads to a signal blockade via VEGF receptors (including both VEGFR-1 (Flt1) and VEGFR-2 (flk1, kinase insert domain receptor, KDR)) and, subsequently to an inhibition of malignant angiogenesis.

There are reports describing small-molecule inhibitors that affect VEGF/VEGFR signaling by directly competing with the ATP-binding site of the respective intracellular kinase domains. This event leads to the inhibition of VEGFR phosphorylation and, ultimately to the apoptotic death of the aberrant endothelial cells. Drug candidates that exhibit this mechanism of action include PTK 787 (**A**) and ZD 6474 (**B**). These are Phase III and II clinical candidates, respectively, against various cancers.[4,5] The six-membered ring of a phthalazine template in PTK 787 has been successfully replaced with the vf isosteric anthranyl amide derivatives **C** and **D**. Intramolecular hydrogen bonding was suggested to be responsible for the optimal spatial orientation of pharmacophores, similar to the parent PTK 787.[6]

It has been suggested that the essential pharmacophores for the VEGFR-2 activity of phthalazines and their analogues include: (i) [6,6]fused (or related) aromatic system; (ii) *para*- or 3,4-di-substituted aniline function in the position 1 of phthalazine; and (iii) hydrogen bond acceptor (Lewis' base: lone pair(s) of a nitrogen- or oxygen atom(s)) attached to the position 4 via an appropriate linker (aryl or fused aryl group).[4] In order to further assess structural requirements for the dual VEGFR-1/VEGFR-2 activity, we designed a set of molecules that have neither fused phthalazine system (**A**) nor the intramolecular hydrogen bonding (**C**, **D**). In this Letter, we expand upon our initial findings and disclose potent inhibitors of VEGFR-1 and VEGFR-2 kinases based on a substituted 4-(azolylethyl)thiazole-5-carboxamide template derived from the phthalazine core.[7]

The targeted molecules **4**–**34** were accessed by a five-step procedure as shown in Scheme 1. Anions prepared from methyl azoles or azines **1a**–**e** (LDA, THF, −78 °C) were reacted with 4-chloroethyl acetoacetate. The resultant products were purified by distillation in vacuo (**2a**, **c**;

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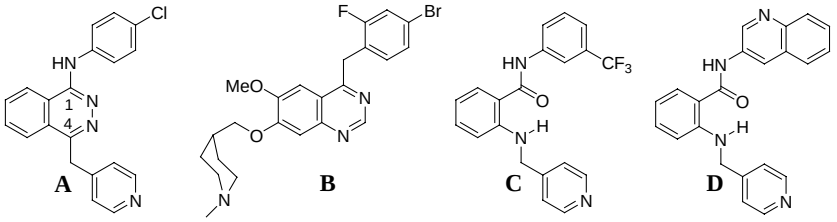


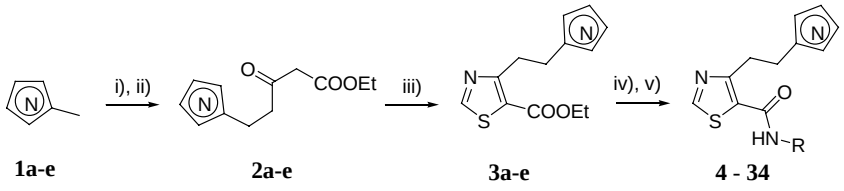
Kugelrohr™) or by column chromatography (**2b**, **d**, and **e**; Silicagel, eluent: EtOAc/hexanes, 1:2) to yield the respective derivatives **2a**–**e** in a 37–58% yield. These were reacted with molecular Br_2 followed by $H_2NC(S)H$ to furnish the respective 4-(azolylethyl)thiazole-5-carboxylates **3a**–**e** in a 54–75% isolated yield. Hydrolysis of the ethyl esters **3a**–**e** with the LiOH/MeOH/H_2O system followed by the BOP-mediated amine coupling afforded the targeted molecules **4**–**34**. The final products were purified by flash chromatography on Silicagel (eluent EtOAc/hexanes, 2:1) to yield analytically pure materials in a 61–88% isolated yield.[8]

Thirty-one compounds (**4**–**34**, Table 1) were tested in vitro against isolated VEGFR-2. Specifically, we measured their ability to inhibit phosphorylation of a biotinylated polypeptide substrate (*p*GAT, CIS Bio International) in a homogeneous time-resolved fluorescence (HTRF) assay at an ATP concentration of 2 μM. The results were reported as a 50% inhibition concentration value (IC_{50}). Literature VEGFR-2 inhibitors **A**–**D** were included as an internal standards for quality control.[6,9]

As seen from Table 1, a number of 4-(azolylethyl)thiazole-5-carboxamides exhibited robust inhibitory activity against VEGFR-2. By varying both 5-carboxamide- and 4-(azolylethyl)-substituents, it was possible to modulate compound potency against the enzyme. Initially, we kept anilinic pharmacophore (4-Cl-C_6H_4)[4] and studied the inhibitory effect of heterocyclic substituents (**1a**–**e**) on the enzymatic activity of the resultant compounds **4**–**13**. 4-Pyridylethyl derivative **6** furnished the best activity with the IC_{50} value of 0.13 μM. Weaker potency was observed for the molecules derived from **a** and **d**. Following these data, we decided to continue optimization of molecules modified with **a** and **c** groups. Good inhibitory activity of molecules modified with the 4-pyridyl radical **c** was explained by the proper alignment of the 4-substituent, namely pyridine-type nitrogen atom of a heterocycle (Lewis' base: hydrogen bond acceptor), with the *Arg*1302 moiety in the ATP-binding pocket of VEGFR-2. MMFF94 force field minimization studies suggest a good overlap between series described in this paper and the development candidates PTK787 and **C** (Fig. 1).[10]

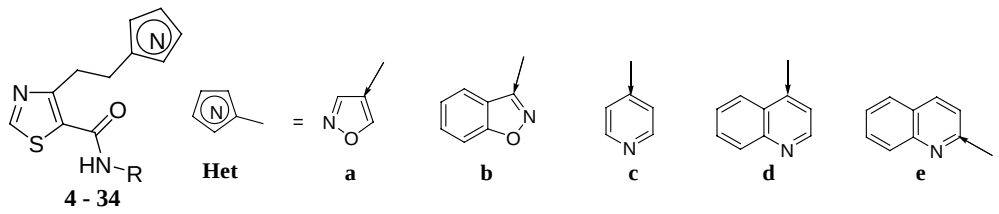
In the next step, we focused on studying SAR of the *amide* portion of the molecule. The molecules substituted with *p*-Cl-, *p*-*t*-Bu-, and *p*-*i*-Pr- groups displayed IC_{50} values of 0.11–0.14 μM in the enzymatic assay (e.g., **6**, **11**, and **15**, Table 1). The (difluorochloro)methoxy group (ClF_2CO-, compound **17**, IC_{50} = 0.12 μM) was also beneficial for the VEGFR-2 inhibition. Small *meta*-substituents on the anilinic portion of the molecule were tolerated (**19**, IC_{50} = 0.56 μM). Similar *ortho*-substitution abolished enzymatic inhibition (**20**, **21**; IC_{50} > 10 μM for both). Several di-substituted aniline fragments, for example, 3,4-di-Cl- (**24**, **25**; IC_{50} = 1.25 and 0.18 μM, respectively), 4-Cl-3-CF_3- (**26**, **27**, IC_{50} = 2.12 and 0.27 μM, respectively), 2-F-4-Me- (**29**; IC_{50} = 0.31 μM, respectively), and 3,4-methylenedioxy groups (**30**; IC_{50} = 0.37 μM) also yielded potent compounds. Related *ortho*-fluoro aminoaryl group has been exemplified in other VEGFR-2 inhibitors such as ZD 6474.[5] Larger hydrophobic 4-substituents on the 5-carboxamide portion of the molecule led to a diminished potency against the enzyme (**31**–**34**). For example, 4-Br derivative (**31**) lost almost 7-fold of activity compared to that of the 4-Cl analogue **6**. Phenyl, phenoxy, and benzyl derivatives (**32**–**34**) showed only moderate enzymatic potency. We speculated that these functions cannot be properly accommodated in the tight hydrophobic pocket of VEGFR-2.[9] Similar observation has been reported by other group.[4]

All compounds were also tested in a HTRF format against VEGFR-1. The results in Table 1 indicate that VEGFR-2 active *N*-(aryl)-4-(azolylethyl)thiazole-5-carboxamides display good activity against VEGFR-1 as well. For the most potent compounds, the IC_{50} values were in the 0.37–0.54 μM range. This outcome could be of benefit in the clinical setting as both receptors are reported to mediate VEGF signaling in the angiogenesis.[11] *Notably, several compounds containing bulky lipophilic substituents at the 5-carboxamide pharmacophore (**11**, **15**, and **17**) yielded 6- to 7-fold selectivity for the VEGFR-2 versus VEGFR-1 kinase*. This observation suggests that it is possible to develop VEGFR-2 specific inhibitors decoupled from the VEGFR-1 activity. Cross-



Scheme 1. Reagents and conditions: (i) LDA, THF, −78 °C, 30 min; (ii) $ClCH_2C(O)CH_2CO_2Et$, 4 h; AcOH/H_2O; (iii) NaH, THF, 0 °C, 30 min to rt, 1 h; $H_2NC(S)H$, MeOH, reflux, 2 h; (iv) LiOH, MeOH, H_2O; (v) R-NH_2, BOP, *i*-Pr_2NEt, CH_2Cl_2, 4 h.

Table 1. Activity of *N*-(aryl)-4-(azolylethyl)thiazole-5-carboxamides against VEGFR-2



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Compound	R	Het	VEGFR-2, enzymatic, IC_{50} (μM)[a,b] obsd (lit)[6]	VEGFR-1, enzymatic, IC_{50} (μM)[a,b] obsd (lit)[6]	VEGFR-2, cell-based ELISA, IC_{50},μM[c] obsd (lit)[6 a]
A, **PTK787**			0.054 ± 0.006 (0.042 ± 0.003)	0.14 ± 0.02 (0.11 ± 0.03)	0.021 ± 0.03 (0.016 ± 0.001)
B, **ZD6474**			0.022 ± 0.003 (0.017 ± 0.003)	0.10 ± 0.01 (0.09 ± 0.01)	1.66 ± 0.11 (2.70 ± 0.17)
C			0.032 ± 0.005 (0.023 ± 0.006)	0.17 ± 0.05 (0.130 ± 0.081)	0.09 ± 0.01 (0.0012 ± 0.0002)
D			0.015 ± 0.004 (0.009 ± 0.001)	0.16 ± 0.05 (0.13 ± 0.03)	0.05 ± 0.01 (0.0012 ± 0.0001)
4	4-Cl(C_6H_4)	**a**	1.85 ± 0.35	4.60 ± 0.65	2.30 ± 0.55
5	4-Cl(C_6H_4)	**b**	>10	>10	>10
6	4-Cl(C_6H_4)	**c**	0.13 ± 0.01	0.31 ± 0.04	0.095 ± 0.01
7	4-Cl(C_6H_4)	**d**	3.38 ± 0.34	6.85 ± 0.64	>10
8	4-Cl(C_6H_4)	**e**	>10	>10	>10
9	4-*t*-Bu(C_6H_4)	**a**	1.45 ± 0.32	>10	1.63 ± 0.46
10	4-*t*-Bu(C_6H_4)	**b**	>10	>10	>10
11	4-*t*-Bu(C_6H_4)	**c**	0.11 ± 0.01	0.72 ± 0.08	0.073 ± 0.01
12	4-*t*-Bu(C_6H_4)	**d**	2.51 ± 0.42	5.46 ± 0.55	>10
13	4-*t*-Bu(C_6H_4)	**e**	>10	>10	>10
14	4-*i*-Pr(C_6H_4)	**a**	1.61 ± 0.46	6.41 ± 0.57	ND
15	4-*i*-Pr(C_6H_4)	**c**	0.14 ± 0.02	0.84 ± 0.09	0.12 ± 0.03
16	4-ClF_2CO(C_6H_4)	**a**	1.77 ± 0.31	>10	2.25 ± 0.37
17	4-ClF_2CO(C_6H_4)	**c**	0.11 ± 0.02	0.76 ± 0.08	0.085 ± 0.01
18	3-Me(C_6H_4)	**a**	>10	>10	>10
19	3-Me(C_6H_4)	**c**	0.56 ± 0.08	4.66 ± 0.54	ND
20	2-Me(C_6H_4)	**a**	>10	>10	ND
21	2-Me(C_6H_4)	**c**	>10	>10	ND
22	4-*N*-Morpholino-(C_6H_4)	**a**	3.68 ± 0.48	>10	ND
23	4-*N*-Morpholino-(C_6H_4)	**c**	0.71 ± 0.08	2.33 ± 0.35	0.58 ± 0.05
24	3,4-Cl(C_6H_4)	**a**	1.25 ± 0.22	5.42 ± 0.56	ND
25	3,4-Cl(C_6H_4)	**c**	0.18 ± 0.02	0.59 ± 0.07	0.093 ± 0.01
26	4-Cl-3-CF_3(C_6H_3)	**a**	2.12 ± 0.33	5.32 ± 0.57	ND
27	4-Cl-3-CF_3(C_6H_3)	**c**	0.27 ± 0.03	0.72 ± 0.09	0.19 ± 0.03
28	2-F-4-Me(C_6H_3)	**a**	1.66 ± 0.25	3.02 ± 0.45	2.75 ± 0.53
29	2-F-4-Me(C_6H_3)	**c**	0.31 ± 0.05	0.63 ± 0.06	0.23 ± 0.02
30	3,4-methylenedioxy	**c**	0.37 ± 0.02	0.75 ± 0.09	0.33 ± 0.04
31	4-Br(C_6H_4)	**c**	0.86 ± 0.11	3.85 ± 0.62	1.02 ± 0.08
32	4-Ph(C_6H_4)	**c**	1.08 ± 0.18	>10	ND
33	4-PhO(C_6H_4)	**c**	1.92 ± 0.43	>10	ND
34	4-Bn(C_6H_4)	**c**	>10	>10	ND

[a] IC_{50} values were determined from the logarithmic concentration-inhibition point (ten points). The important values are given as means of at least two duplicate experiments; ND, not determined.

[b] Lit IC_{50} values, as measured at 8 μM ATP.

[c] Literature data correspond to the inhibition of VEGF-induced phosphorylation of VEGFR-2 in CHO cells.

reactivity screening of **4**–**34** against a number of other receptors (IGF1R, InR, FGFR1, Flt3, ErbB1, ErbB2, EphB4, and c-Met) and cytosolic (PKA, GSK3β, PKB/Akt, bcr-Abl, and Cdk2/5) kinases in an HTRF format indicated no significant cross-reactivity (PI > 40%, triplicate measurements) at a screening concentration of 10 μM.

Active in vitro inhibitors of VEGFR-2 were further characterized in a cell-based phosphorylation ELISA (Table 1).[12] In general, good in vitro-to-cell based activity correlation has been found for these compounds. In our hands, the best compounds displayed 73–93 nM activity in inhibiting cell-based phosphorylation of VEGFR-2. This fact indicates that a number of thiazole molecules, including **6**, **11**, **17**, and **25**, could be further developed for in vivo studies.

In summary, we have described a series of *N*-(aryl)-4-(azolylethyl)thiazole-5-carboxamides as potent inhibitors of the VEGFR-2 receptor in both in vitro and cell-based assays (IC_{50} > 100 nM). By controlling the substitution pattern on the 5-carboxamido pharmacophore, both dual and specific VEGFR-2 thiazoles were

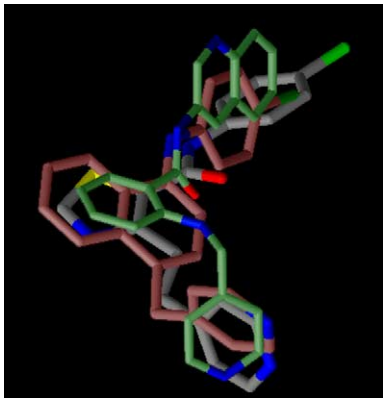

Figure 1. Structural overlap between our inhibitor (compounds **6**, gray), PTK787 (**A**, brown), and **C** (green).

identified. The analogues presented in this *Letter* are potentially useful in the treatment of conditions such as cancer. Further details on their biological properties, such as functional activity, together with murine oral exposure data will be presented in due course.

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8. *Analytical data for* **6**. *N*-(4-Chlorophenyl)-4-(2-(pyridin-4-yl)ethyl)thiazole-5-carboxamide; mp 189–191 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.56 (t, J = 7.6 Hz, 2H), 2.91 (t, J = 7.6 Hz, 2H), 7.22 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 8.54 (d, J = 8.0 Hz, 2H), 9.22 (s, 1H), 13.10 (br s, exch. D_2O, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 26.9, 35.2, 118.3, 122.6, 123.0, 129.5, 130.1, 133.6, 145.2, 147.9, 149.1, 153.2, 163.1; ESI MS (M+1): 345, (M−1): 343; HRMS, exact mass calcd. for $C_{17}H_{14}ClN_3OS$: 343.0546, found: 343.0538. Elemental analysis: Calcd for $C_{17}H_{14}ClN_3OS$: C, 59.38; H, 4.10; N, 12.22. Found: C, 59.23; H, 4.21, N, 12.04.
9. VEGFR-2 kinase inhibition was determined by measuring the phosphorylation level of *poly*-Glu-Ala-Tyr-biotin (*p*GAT-biotin) peptide in the HTRF assay. Into a 96-well Costar plate was added 2 μl/well of 25× compound in a 100% DMSO (final concentration in the 50 μl kinase reaction is typically 1 nM to 10 μM). Next, 38 μl of reaction buffer (25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM DTT, and 1 mg/ml BSA) containing 0.5 mmol *p*GAT-biotin and 3–4 ng KDR enzyme was added to each well. After 5–10 min preincubation, the kinase reaction was initiated by the addition of 10 μl of 10 μM ATP in the reaction buffer, after which the plate was incubated at room temperature for 45 min. The reaction was stopped by addition of 50 μl KF buffer (50 mM Hepes, pH 7.5, 0.5 M KF, and 1 mg/ml BSA) containing 100 mM EDTA and 0.36 μg/ml PY20K (Eu-cryptate labeled anti-phosphotyrosine antibody, CIS Bio International) was added and after an additional 2 h incubation at RT, the plate was analyzed in a RUBYstar HTRF Reader.
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12. Cell-based assay for VEGFR-2 inhibition: (i) *Transfection of 293 cells with DNA expressing FGFR1/VEGFR-2 chimera*: A chimeric construct containing the extracellular portion of FGFR1 and the intracellular portion of VEGFR-2 was transiently transfected into 293 adenovirus-transfected kidney cells. DNA for transfection was diluted to a 5 μg/ml final concentration in a serum-free medium and incubated at room temperature for 30 min with 40 μl/ml of Lipofectamine 2000, also in serum-free media. Two hundred and fifty microliters of the Lipofectamine/DNA mixture was added to 293 cells suspended at 5×10^5 cells/ml. 200 μl/well of the suspension was added to a 96-well plate and incubated overnight. Within 24 h, media were removed and 100 μl of media with 10% fetal bovine serum was added to the now adherent cells followed by an additional 24 h incubation. Test compounds were added to the individual wells (final DMSO concentration was 0.1%). Cells were lysed by re-suspension in 100 μl Lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.5, 0.5% Triton X-100, 10 mM NaPPi, 50 mM NaF, and 1 mM Na_3VO_4) and rocked for 1 h at 4 °C. (ii) *ELISA for detection of tyrosine-phosphorylated chimeric receptor*: 96-well ELISA plates were coated using 100 μl/well of 10 μg/ml αFGFR1 antibody and incubated overnight at

4 °C. αFGFR1 was prepared in a buffer made with 16 ml of a 0.2 M Na_2CO_3 and 34 ml of a 0.2 M $NaHCO_3$ with pH adjusted to 9.6. Concurrent with lysis of the transfected cells, αFGFR1-coated ELISA plates were washed three times with PBS + 0.1% Tween 20, blocked by addition of 200 μl/well of a 3% BSA in PBS for 1 h, and washed again. Eighty microliters of lysate was then transferred to the coated and blocked wells and incubated for 1 h at 4 °C. The plates were washed three times with PBS + 0.1% Tween 20. To detect bound phosphorylated chimeric receptor, 100 μl/well of anti-phosphotyrosine antibodies (RC20:HRP, Transduction Laboratories) was added (final concentration 0.5 μg/ml in PBS) and incubated for 1 h. The plates were washed six times with PBS + 0.1% Tween 20. Enzymatic activity of HRP was detected by adding 50 μl/well of equal amounts of the Kirkegaard & Perry Laboratories (KPL) Substrate A and Substrate B. The reaction was stopped by addition of 50 μl/well of a 0.1 N H_2SO_4 and absorbance was measured at 450 nm.