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Hetaryl imidazoles: A novel dual inhibitors of VEGF receptors I and II

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Abstract—A novel potent derivatives of hetaryl imidazoles were 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 functionality, both dual and specific VEGFR-2 thiazoles were identified.

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 developed methods for sequestering VEGF. This leads to 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 domain. 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 replaced with the 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; (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 this *Letter*, we expand upon our initial findings and disclose potent inhibitors of VEGFR-2 kinase based on a substituted imidazole core.[7] We reasoned that this template could provide for the proper pharmacophore arrangement relevant to compounds **A**, **C**, and **D**.

The targeted molecules **5**–**31** were accessed by a procedure shown in Scheme 1. Commercially available imidazole **1** was heated in a HNO_3/H_2SO_4 mixture (5:6) at 70 °C for 3 h. The resulting 4-nitro-5-chloro derivative was treated with the KCN/KI system in EtOH at reflux to yield **2**, a product of S_NAr of Cl, in 43% overall isolated yield. The nitrile function was hydrolyzed by heating **2** in aqueous H_2SO_4 at 80 °C overnight to afford the respective acid **3** in 73% yield. The acid **3** was converted to a series of 1,3,4-oxadizoles **4** (55–74% overall yield) via the published route.[8] The synthetic sequence

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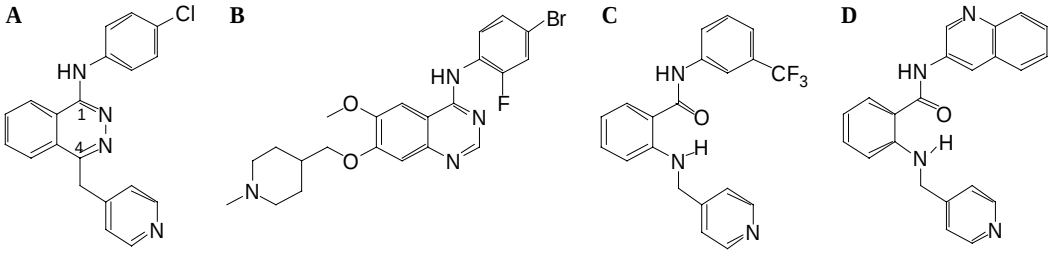


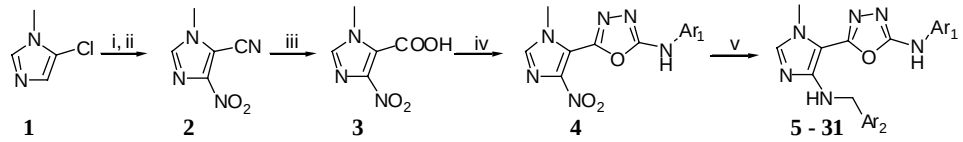
involved the formation of acid hydrazide, its reaction with aryl thiocyanate, and DCC-promoted heterocyclization of the respective thiosemicarbazone derivative in refluxing toluene.[9] The nitro group was further reduced to the respective amine by hydrogenation over a 10% Pd/C in an EtOAc/DMF mixture to insure for the solubility of **4**. The resulting amines were reacted with a series of aldehydes in dry MeOH to yield the respective Schiff' bases. These were reduced to the targeted aryl methylamines **5**–**31** with $NaBH_4$ in 39–58% isolated yields.

Twenty-seven compounds (**5**–**31**, Table 1) were tested in vitro against isolated VEGFR-2 kinase. 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** (Table 1) were included as internal standards for quality control.[10]

As seen from Table 1, a number of imidazole derivatives exhibited robust inhibitory activity against VEGFR-2. By varying both aryl substituents (anilinic groups) on a 1,3,4-oxazole function and aryl methylamine pharmacophores, we modulated compound potency against the enzyme. Initially, we kept the anilinic function (4-Cl-C_6H_4)[4] constant and studied the inhibitory effect of an aryl methylamine group on the enzymatic activity of the resultant compounds **5**–**12** (Table 1). 4-Pyridylmethyl derivative **5** furnished the best activity with the IC_{50} value of 0.25 μM against VEGFR-2. Weaker potency was observed for the molecule **8** derived from piperonal (IC_{50} = 0.96 μM). Following these data, we decided to continue optimization of molecules derived from 4-pyridyl aldehyde (**13**–**31**, Table 1). Good inhibitory activity of **5** 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. We further reasoned that in **6** and **7** the nitrogen of the pyridine ring is likely to be misaligned with the *Arg*1302. Similarly, indazole and quinoline functions in **10**–**12** could be too large to fit into a respective binding pocket.[11]

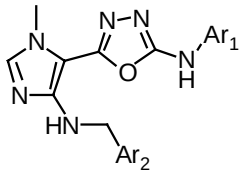
In the next step, we focused on studying SAR of the anilinic portion of the molecule attached to the 1,3,4-oxadiazole moiety. The molecules substituted with *p*-Cl-, *p*-*t*-Bu-, and *p*-*i*-Pr-groups displayed moderate potency with the IC_{50} values of 0.25–0.44 μM in the enzymatic assay (e.g., **5**, **13**, and **14**). The 4-ClF_2CO-group (**15**) resulted in improved potency (IC_{50} = 0.13 μM). 4-F_3CO- and 4-F_3C-derivatives (**16**, **17**) also showed good activity against VEGFR-2 (IC_{50}s of 93 and 73 nM). Both 3-F_3CO- (**18**) and 3-MeO- (**20**) derivatives led to enzymatic potencies of 44 and 130 nM, respectively. Small *meta*-substituents were considerably less active (**19**, IC_{50} = 0.77 μM). Similar *ortho*-substitution abolished enzymatic inhibition (**20**, **21**; IC_{50} > 10 μM for both). 3,4-Di-substituted aryl groups also yielded potent compounds. Examples include 3,4-di-Cl- (**23**; IC_{50} = 0.18 μM), 3,4-*di*-MeO- (**24**, IC_{50} = 52 nM), 4-Cl-3-CF_3- (**25**, IC_{50} = 43 nM), and piperonyl groups (**27**; IC_{50} = 0.14 μM). Notably, 2-F-4-Me-group was well tolerated (**26**; IC_{50} = 0.26 μM). Related *ortho*-fluoro aniline group has been exemplified in other VEGFR-2 inhibitors such as ZD 6474.[5] Larger anilinic substituents led to the diminished potency against the enzyme (**28**–**31**). For example, 4-Br-derivative (**28**) lost ca. 3-fold activity compared to the 4-Cl analogue **5**. Phenyl, phenoxy, and benzyl derivatives (**29**–**31**) showed moderate to no enzymatic activity. We speculated that these functions cannot be properly accommodated in the tight hydrophobic pocket of VEGFR-2.[11]

All compounds were also tested in an HTRF format against VEGFR-1. The results in Table 1 indicate that VEGFR-2 active imidazoles display good activity against VEGFR-1 as well. For the most potent compounds, the IC_{50} values were in the 0.12–0.59 μM range.



Scheme 1. Reagents and conditions: (i) HNO_3, H_2SO_4, H_2O, 90 °C, 2 h; (ii) KCN, KI, EtOH, reflux, 8 h; (iii) H_2SO_4, EtOH, 80 °C, 8 h; (iv) NH_2NH_2, H_2O, *i*-PrOH, reflux, 4 h; Ar_1NCS, CH_2Cl_2, 2 h; DCC, toluene, reflux, 4 h; (v) H_2, Pd/C, EtOAc/DMF; (vi) Ar_2CHO, MeOH; $NaBH_4$, *i*-PrOH, reflux, 2 h.

Table 1. Activity of hetaryl imidazoles **5**–**31** against VEGFR-2



5-31

Compound	Ar_1	Ar_2	VEGFR-2, enzymatic, IC_{50} (μM)[a]	VEGFR-1, enzymatic, IC_{50} (μM)[a]	VEGFR-2, cell-based ELISA, IC_{50} (μM)[a]
A, PTK787			0.054 ± 0.006	0.14 ± 0.02	0.021 ± 0.03
			(0.042 ± 0.003)	(0.11 ± 0.03)	(0.016 ± 0.001)
B, ZD6474			0.022 ± 0.003	0.10 ± 0.01	1.66 ± 0.11
			(0.017 ± 0.003)	(0.09 ± 0.01)	(2.70 ± 0.17)
C			0.032 ± 0.005	0.17 ± 0.05	0.09 ± 0.01
			(0.023 ± 0.006)	(0.130 ± 0.081)	(0.0012 ± 0.0002)
D			0.015 ± 0.004	0.16 ± 0.05	0.05 ± 0.01
			(0.009 ± 0.001)	(0.13 ± 0.03)	(0.0012 ± 0.0001)
5	4-Cl(C_6H_4)	4-Pyridine	0.25 ± 0.03	0.34 ± 0.04	0.57 ± 0.08
6	4-Cl(C_6H_4)	3-Pyridine	>10	>10	>10
7	4-Cl(C_6H_4)	2-Pyridine	>10	>10	>10
8	4-Cl(C_6H_4)	Piperonyl	0.96 ± 0.08	1.47 ± 0.18	>10
9	4-Cl(C_6H_4)	3,4-Di-F(C_6H_3)	1.86 ± 0.15	3.31 ± 0.26	5.65 ± 0.72
10	4-Cl(C_6H_4)	5-Indazole	2.37 ± 0.34	>10	>10
11	4-Cl(C_6H_4)	2-Quinoline	>10	>10	>10
12	4-Cl(C_6H_4)	5-Quinoline	3.54 ± 0.44	>10	>10
13	4-*t*-Bu(C_6H_4)	4-Pyridine	0.38 ± 0.04	0.89 ± 0.08	1.05 ± 0.11
14	4-*i*-Pr(C_6H_4)	4-Pyridine	0.44 ± 0.07	0.77 ± 0.06	1.15 ± 0.12
15	4-ClF_2CO(C_6H_4)	4-Pyridine	0.13 ± 0.01	0.46 ± 0.06	0.096 ± 0.01
16	4-F_3CO(C_6H_4)	4-Pyridine	0.093 ± 0.01	0.16 ± 0.02	0.066 ± 0.01
17	4-F_3C(C_6H_4)	4-Pyridine	0.073 ± 0.01	0.12 ± 0.03	0.082 ± 0.009
18	3-F_3CO(C_6H_4)	4-Pyridine	0.046 ± 0.008	0.56 ± 0.07	0.13 ± 0.01
19	3-Me(C_6H_4)	4-Pyridine	0.71 ± 0.09	1.26 ± 0.11	ND
20	3-MeO(C_6H_4)	4-Pyridine	0.13 ± 0.02	0.67 ± 0.08	0.064 ± 0.01
21	2-MeO(C_6H_4)	4-Pyridine	>10	>10	ND
22	2-Me(C_6H_4)	4-Pyridine	>10	>10	ND
23	3,4-Di-Cl(C_6H_4)	4-Pyridine	0.18 ± 0.02	0.49 ± 0.05	0.26 ± 0.05
24	3,4-Di-MeO(C_6H_4)	4-Pyridine	0.052 ± 0.009	0.59 ± 0.07	0.093 ± 0.01
25	4-Cl-3-CF_3(C_6H_3)	4-Pyridine	0.043 ± 0.008	0.51 ± 0.06	0.10 ± 0.01
26	2-F-4-Me(C_6H_3)	4-Pyridine	0.26 ± 0.04	0.59 ± 0.06	0.64 ± 0.09
27	Piperonyl-	4-Pyridine	0.14 ± 0.02	0.44 ± 0.05	0.27 ± 0.03
28	4-Br(C_6H_4)	4-Pyridine	0.73 ± 0.13	1.47 ± 0.34	ND
29	4-Ph(C_6H_4)	4-Pyridine	3.22 ± 0.32	>10	ND
30	4-PhO(C_6H_4)	4-Pyridine	>10	>10	ND
31	4-Bn(C_6H_4)	4-Pyridine	>10	>10	ND

ND = not determined.

[a] IC_{50} values were determined from the logarithmic concentration-inhibition point (10 points). The important values are given as means of at least two duplicate experiments.

This outcome could be of benefit in the clinical setting as both receptors are reported to mediate VEGF signaling in angiogenesis.[12] Notably, several compounds containing bulky *meta*- and 3,4-di-substituted anilinic functions at the 1,3,4-oxadiazole pharmacophore (**18**, **24**, and **25**) yielded over 10-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. Structural reasons for this VEGFR-2 specificity are under further investigation. Cross-reactivity screening of **5**–**31** against a number of other receptor (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 < 30%, 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).[13] In general, good in vitro-to-cell-based activity correlation has been found for these compounds. In our hands, the best compounds displayed 66–96 nM activity in inhibiting cell-based phosphorylation of VEGFR-2. This fact indicates that a number of imidazole derivatives, including **15**–**17**, **20**, and **24**, could be further developed for in vivo studies. All active compounds showed good solubility (>5 mM) in both water and screening media, as evidenced by HPLC experiments.

In summary, we have described a series of novel imidazole derivatives 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 anilinic portion of the molecule, both dual and specific VEGFR-2 imidazoles were 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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9. *Analytical data for selected compounds*. Compound **5**: *N*-(4-chlorophenyl)-5-(1-methyl-4-(pyridin-4-ylmethylamino)-1*H*-imidazol-5-yl)-1,3,4-oxadiazol-2-amine; mp 229–231 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 3.55 (s, 3H), 4.25 (d, J = 7.4 Hz, 2H), 4.61 (br s, exch. D_2O, 1H, NH), 5.06 (br s, exch. D_2O, 1H, NH), 6.88 (d, J = 8.0 Hz, 2H), 7.12 (d, J = 8.0 Hz, 2H), 7. 55 (d, J = 8.4 Hz, 2H), 8.56 (d, J = 8.4 Hz, 2H), 8.92 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 27.8, 45.7, 116.5, 123.7, 124.3, 125.9, 127.4, 128.2, 129.6, 133.2, 138.1, 142.0, 146.8, 150.2; ESI MS (M+1): 383, (M−1): 381; HRMS, exact mass calcd for $C_{18}H_{16}ClN_7O$: 381.1105, found: 381.1093. Elemental analysis: calcd for $C_{18}H_{16}ClN_7O$: C, 56.62; H, 4.22; N, 25.68. Found: C, 56.41; H, 4.35, N, 25.54.Compound **8**: 5-(4-(benzo[*d*][1,3]dioxol-5-ylmethylamino)-1-methyl-1*H*-imidazol-5-yl)-*N*-(4-chloro phenyl)-1,3,4-oxadiazol-2-amine; mp 196–198 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 3.59 (s, 3H), 4.13 (d, J = 7.4 Hz, 2H), 4.68 (br s, exch. D_2O, 1H, NH), 5.12 (br s, exch. D_2O, 1H, NH), 5.88 (s, 2H), 6.53 (m, 2H), 6.62 (d, J = 6.8 Hz, 1H), 6.84 (d, J = 8.0 Hz, 2H), 7.12 (d, J = 8.0 Hz, 2H), 8.91 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 28.1, 46.0, 101.4, 111.6, 114.9, 117.0, 120.1, 123.3, 124.5, 125.9, 126.7, 127.1, 129.3, 135.2, 138.4, 141.0, 146.2, 148.3; ESI MS (M+1): 426, (M−1): 424; HRMS, exact mass calcd for $C_{20}H_{17}ClN_6O_3$: 424.1051, found: 424.1043. Elemental analysis: calcd for $C_{20}H_{17}ClN_6O_3$: C, 56.54; H, 4.03; N, 19.78. Found: C, 56.31; H, 3.88, N, 19.56.
10. 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 per 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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13. *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 Lipofectamine 2000, also in serum-free media. Twenty-five microliters of the Lipofectamine/DNA mixture was added to 293 cells suspended at 5×10^5 cells/ml. Two hundred microliters per 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: Ninety-six-well ELISA plates were coated using 100 μl per 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 per well of a 3% BSA in PBS for 1 h, and washed again. Eight 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 per 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 per well of equal amounts of the Kirkegaard & Perry Laboratories (KPL) Substrate A and Substrate B. The reaction was stopped by addition of 50 μl per well of a 0.1 N H_2SO_4, and absorbance was measured at 450 nm.