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Arylphthalazines: Identification of a new phthalazine chemotype as inhibitors of VEGFR kinase

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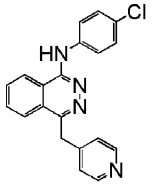
Abstract—A novel class of 4-arylamino-phthalazin-1-yl-benzamides is described as inhibitors of vascular endothelial growth factor receptor II (VEGFR-2). Several compounds display potent VEGFR-2 inhibitory activity with an IC_{50} as low as 0.078 μM in an HTRF enzymatic assay. These compounds are relatively selective against a small kinase panel.

Angiogenesis, the formation of new vasculature from an existing vascular network, is an important physiological process that is involved in embryonic development, follicular growth, and wound healing, as well as pathological conditions such as cancer.[1] Although angiogenesis is a highly complex process, there is a large body of evidence that suggests that an endothelial cell-specific mitogen, vascular endothelial growth factor (VEGF), is a major regulator of these events.[2] The biological effects of VEGF are mediated by two receptor tyrosine kinases known as VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as KDR in humans or flk1 in mice). The present understanding seems to indicate that the effect of VEGF binding to VEGFR-2 is the major mediator of vascular endothelial cell mitogenesis, angiogenesis, and microvascular permeability,[3] and inhibitors of VEGFR-2 have therefore become a major focus of many research organizations. One such approach for obtaining VEGFR-2 inhibition involves using small organic molecules to block adenosine triphosphate (ATP) binding to the intracellular kinase domain of the receptor, resulting in diminished VEGF signal transduction. Numerous compounds, such as PTK 787 (**1**) and ZD 6474 (**2**), have been shown to be effective in this manner,[4,5] with these and other molecules progressing to the clinic for further evaluation. In this Letter, we disclose some of our own work toward inhibition of VEGFR-2 with small molecules and detail a new class of phthalazine compounds that act at this receptor.[6]



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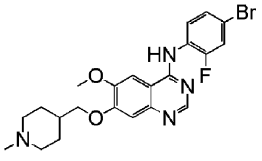

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A screening campaign examining the ability of compounds to block the phosphorylation of a biotinylated polypeptide substrate (*p*-GAT, CIS bio international) in a homogeneous time-resolved fluorescence (HTRF) assay,[7] at an ATP concentration of 2 μM, identified 4-{4-[4-(chloro-difluoromethoxy)-phenylamino]-phthalazin-1-yl}-benzamide (**3**) as an inhibitor of VEGFR-2 (IC_{50} = 0.19 μM, Table 1). To evaluate this class of compounds further, a number of analogs were synthesized according to the details in Scheme 1. Thus,

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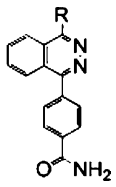
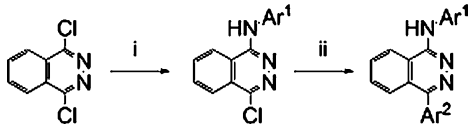
Table 1. Enzymatic activities of (**3**–**23**) toward VEGFR-2 kinase[7]



Compound	R	Enzymatic, IC_{50} (μM)[a]
3	NH(4-OCF_2Cl)Ph	0.19 ± 0.05
4	NH(3-*t*-Bu)Ph	0.47 ± 0.07
5	NH(4-*t*-Bu)Ph	0.078 ± 0.02
6	NH(4-Br)Ph	0.1 ± 0.03
7	NH(3,4-Cl_2)Ph	0.84 ± 0.26
8	NH(3-Cl)Ph	1.7 ± 0.2
9	NH(3-Me)Ph	1.6 ± 0.22
10	NH(3-Br)Ph	1.2 ± 0.1
11	NH(4-Cl)Ph	1.3 ± 0.1
12	NH(4-Me)Ph	1.8 ± 0.2
13	NH(4-OMe)Ph	2.0 ± 0.3
14	NH(4-CO_2Me)Ph	2.7 ± 0.7
15	NH Ph	>10
16	NH(2,4-Me_2)Ph	6.9 ± 1.1
17	NH(4-$CONH_2$)Ph	>10
18	NH(4-SO_2NH_2)Ph	>10
19	NH(4-OBn)Ph	>10
20	Morpholin-4-yl	>10
21	Piperidin-1-yl	>10
22	O (4-OCF_3)Ph	>10
23	S (4-OCF_3)Ph	>10

[a] IC_{50} values were determined from logarithmic concentration–inhibition curves (at least eight points) and are given as means of at least two separate experiments.



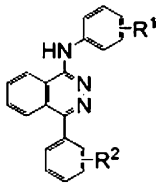
Scheme 1. Reagents and conditions: (i) Ar^1NH_2, Et_3N, ^nBuOH, 100 °C; (ii) $Ar^2B(OH)_2$, K_2CO_3, $PdCl_2(Ph_3P)_2$, 1,4-dioxane, H_2O, μwave, 100 °C.

commercially available 1,4-dichlorophthalazine was first reacted with an amine to give the mono-substituted adduct.[8] The remaining chloride was then coupled with a boronic acid or a boronic acid ester under Suzuki reaction conditions with microwave irradiation to give the desired final compound. In addition, further examples of our design were prepared by a custom synthesis supplier.[9]

As can be seen from Table 1, phthalazine derivatives incorporating a 4-carbamoylphenyl group were effective at inhibiting VEGFR-2. For example, compounds (**3**–**7**) displayed submicromolar activity against VEGFR-2. Other substituents present in the aromatic amine that appear to be tolerated include the *meta*-chloro, *meta*-methyl, *meta*-bromo, *para*-chloro, *para*-methyl, *para*-methoxy, and *para*-methoxycarbonyl groups (**8**–**14**). Significantly, an absence of substitution, or *ortho*-substitution of the aromatic amine with a methyl group, resulted in compounds that were poorly active against VEGFR-2 (**15** and **16**). In addition, a number of other congeners were also not active (**17**–**19**). Compounds (**3**–**19**) differ in their substitution of a phenyl ring, which is believed to occupy a hydrophobic pocket in the VEGFR-2 kinase domain. Many of the substituents active in our compounds have also been noted as favorable groups in a related class of phthalazine VEGFR-2 inhibitors.[4] Finally, replacement of the aromatic amine of (**3**) with a tertiary aliphatic amine, or with an aromatic ether, or thioether resulted in the abolition of VEGFR-2 inhibition (**20**–**23**). These observations suggest the importance of an aromatic secondary amino group that may participate in hydrogen bonding interactions at the active site. Similar results were observed for related phthalazine scaffolds where a secondary amino group is a crucial element for binding at the VEGFR-2 kinase active site.[4]

As can be seen from Table 2, the amide of the lead series could be moved to the *meta*-position of the phenyl ring, although inhibitory activity against VEGFR-2 was somewhat diminished (compare compounds **24**

Table 2. Enzymatic activities of (**24**–**48**) toward VEGFR-2 kinase[7]



Compound	R^1	R^2	Enzymatic, IC_{50} (μM)[a]
24	4-*t*-Bu	3-$CONH_2$	0.40 ± 0.11
25	4-OCF_2Cl	3-$CONH_2$	4.1 ± 0.3
26	4-*t*-Bu	4-CONHMe	0.13 ± 0.02
27	4-Cl	4-CONHMe	3.1 ± 0.4
28	4-Me	4-$CONMe_2$	>10
29	4-OCF_2Cl	4-CO-morpholin-4-yl	4.9 ± 1.2
30	4-OCF_2Cl	4-$CONHCH_2CH_2OH$	1.1 ± 0.5
31	4-OCF_2Cl	4-$CONH^tBu$	>10
32	4-OCF_2Cl	4-$CONH^iPr$	>10
33	4-OCF_2Cl	4-CO_2Me	0.63 ± 0.18
34	4-Cl	4-CO_2Me	0.36 ± 0.02
35	4-OCF_2Cl	4-Me-3-SO_2NH_2	>10
36	4-OCF_2Cl	4-Me-3-SO_2NH^iPr	>10
37	4-OCF_2Cl	4-Me	>10
38	3,4-Cl_2	4-Me	>10
39	4-Cl	4-Me	>10
40	4-OCF_2Cl	4-OMe	>10
41	3,4-Cl_2	4-OMe	>10
42	4-Cl	4-OMe	>10
43	4-OMe	4-OEt	>10
44	4-Cl	4-OEt	>10
45	4-Me	4-OEt	>10
46	4-OCF_2Cl	4-Cl	>10
47	4-OCF_2Cl	H	>10
48	4-OMe	H	>10

[a] IC_{50} values were determined from logarithmic concentration–inhibition curves (at least eight points) and are given as means of at least two separate experiments.

Table 3. Enzymatic activities of selected compounds toward VEGFR-1 and VEGFR-2 kinases[9]

Compound	% Inhibition of VEGFR-1 at 10 μM[a]	% Inhibition of VEGFR-2 at 10 μM[a]
4	60	86
5	73	93
6	53	71
10	51	74

[a] Average of $n = 3$.

and **25** to **5** and **3**). Mono-methyl substitution at the amide group was tolerated, although the activity seems to be reduced in some cases (**26** and **27**), whilst bis-methylation at the amide group resulted in an inactive compound (**28**). This alkylation strategy could also be extended to include substitution with a group to aid in solubility (**29** and **30**). Substitution with bulkier groups, such as *tert*-butyl and *iso*-propyl, resulted in a complete loss of activity (**31** and **32**), suggesting a relatively restricted binding area. The amide moieties in (**3**) and (**4**) could be replaced by a methoxycarbonyl subunit (**33** and **34**) without significant loss of activity. However, two sulfonamide replacements of the amide resulted in no inhibitory activity against VEGFR-2 (**35** and **36**), although it should be noted that these are disubstituted analogs. Removal of the amide group, or its substitution with another small group (Me, OMe, OEt, Cl) in compounds (**37**)–(**48**), completely eliminated the activity toward VEGFR-2 kinase. Thus, the presence of a carbonyl-containing group (amide or methyl ester) proved to be important for activity in this class of compounds.[10]

A number of compounds described above were also tested against a small kinase cross-reactivity panel including VEGFR-1, a related receptor tyrosine kinase. The compounds showed little or no activity against EGFR, FGF-R1, InsR, c-Met, or IGF-1R at a screening concentration of 10 μM.[11] However, preliminary data indicated a significant inhibitory activity for both VEGFR-1 and VEGFR-2 kinases (Table 3).

In summary, this Letter describes a new class of arylphthalazine compounds as inhibitors of the VEGFR kinase family members.

Acknowledgment

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6. See Lu, D.; Jimenez, X.; Zhang, H.; Bohlen, P.; Witte, L.; Zhu, Z. *Int. J. Cancer* **2002**, *97*, 393, for an antibody approach for VEGFR-2 inhibition.
7. VEGFR tyrosine kinase inhibition is determined by measuring the phosphorylation level of poly-Glu-Ala-Tyr-biotin (pGAT-biotin) peptide in a Homogeneous Time-Resolved Fluorescence (HTRF) assay. Into a black 96-well Costar plate is added 2 μl/well of 25x compound in 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 pGAT-biotin and 3–4 ng KDR enzyme is added to each well. After 5–10 min preincubation, the kinase reaction is initiated by the addition of 10 μl of 10 μM ATP to reaction buffer, after which the plate is incubated at room temperature for 45 min. The reaction is stopped by addition of 50 μl KF buffer (50 mM Hepes, pH 7.5, 0.5 M KF, 1 mg/ml BSA) containing 100 mM EDTA and 0.36 μg/ml PY20K (Eu-cryptate labeled anti-phosphotyrosine antibody, CIS Bio International). After 30 min, 100 μl of 10 nM SV-XL (modified APC-labeled Streptavidin, CIS Bio International) in KF buffer is added, and after an additional 2 h incubation at room temperature, the plate is read in a RUBYstar HTRF Reader.
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9. These compounds were prepared by an alternative route that will be detailed in a full report of this work.
10. An alternative arylphthalazine chemotype lacking the carbonyl moiety was found to be very active against VEGFR-2. Results will be disclosed in due course.
11. Compounds (**3**–**29**) were tested against this small kinase panel. For example, the percentage inhibition for compound (**5**) against these kinases at a screening concentration of 10 μM is as follows: FGF-R1, 36%; c-met, 12%; EGFR, 14%; IGF-1R, 20%; InsR, 25% (average of $n = 3$). A percentage inhibition of <40% at 10 μM is considered to be inactive in our hands.