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Structure-guided identification of novel VEGFR-2 kinase inhibitors via solution phase parallel synthesis

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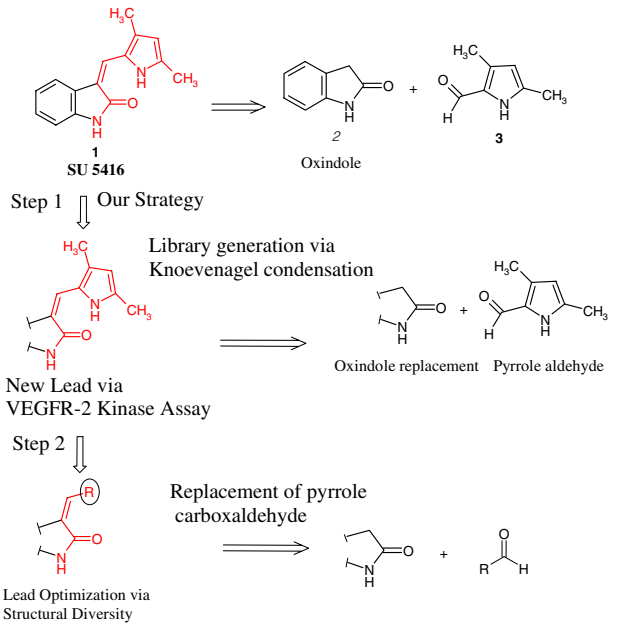
Abstract—Structural analysis of the essential binding elements of the oxindole-based kinase inhibitor (**1**) led to the identification of a novel class of heterocyclic-substituted pyrazolones. Knoevenagel condensation of a variety of activated methylene nucleophiles with indole or pyrrole carboxaldehydes provided a focused library of molecules, each containing elements of kinase pharmacophore probe. Initial screening for VEGFR-2 kinase inhibition eliminated several of the probes. Identification of an active pyrazolone motif and further optimization resulted in several highly potent VEGFR-2 inhibitors with cellular efficacy, anti-angiogenic activity ex vivo in rat aortic ring explant cultures, and oral anti-tumor efficacy in nude mice.

Despite tremendous advancement in the field of combinatorial chemistry relating to drug discovery, skepticism has been expressed at the success rate of generating lead structures against molecular targets from such large chemical libraries.[1] This drawback has resulted in an initiation of efforts to explore more focused libraries with drug-like physical properties and reduced molecular weight.[2] Herein we report, a structure-guided chemical library building process, where step-by-step structural modifications have generated a novel class of vascular endothelial growth factor receptor-2 (VEGFR-2) kinase inhibitors with oral anti-tumor efficacy in nude mice. Examination of a known class of oxindole-based inhibitors (**1**, Scheme 1) identified the key binding elements. Retrosynthetic analysis fragmented these inhibitors into active methylene nucleophiles and aryl aldehydes. Exploiting the classic Knoevenagel condensation[3] introduced structural diversity in a rapid fashion.

Major organic reactions (such as Aldol, Diels–Alder, etc.,) generate typical structural patterns in products they form and these patterns become diagnostic features in retrosynthetic analysis during synthesis of complex substances such as natural products.[4] Additionally, if



Scheme 1. Retrosynthesis of SU 5416 and kinase re-design strategy.

Keywords: VEGFR-2; Kinase; Inhibitors; Pyrazolones; Oxindole; Anti-tumor; Angiogenesis; Knoevenagel condensation; Solution phase parallel synthesis.
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such a structural pattern present in a molecule constitutes an essential recognition motif against a target protein, then retrosynthetic analysis can play a role in a chemical library building process. By following the reactions as depicted by a retrosynthesis, an essential structural motif against that protein can be generated and grafted on different chemical structures simply by varying the reaction components. As a result, architectural diversity of structures with preservation of essential recognition elements and depletion of undesired segments of the original molecule can be achieved.

Vascular endothelial growth factor (VEGF)[5] and the cell surface receptors in human, VEGFR-2 (also known as KDR; kinase domain containing receptor),[6] are considered to play an important role in angiogenesis,[7] which is vital for survival and proliferation of tumor cells.[8] In recent years, several classes of small molecule-based VEGFR-2 kinase inhibitors have emerged as promising anti-angiogenic agents for possible treatment against a wide variety of cancers.[9] One of the pioneering classes of compounds in this area of research belongs to 3-substituted indolin-2-ones, developed in the laboratory of Sugen, Inc. This motif was shown to interact with the kinase at the ATP binding site (X-ray study), where the indoline core occupies the adenine segment of the ATP.[10] Of particular interest is the indolin-2-one, SU 5416 (**1**).[9a] The five-membered ring lactam unit of **1** forms two critical hydrogen bonding interactions with the enzyme. Structure–activity relationship (SAR) of **1** also showed that the exocyclic double bond and substituted pyrrole unit are crucial for kinase inhibitory activity. Retrosynthetic analysis of SU 5416 (**1**, Scheme 1) shows that the essential part of the pharmacophore (i.e., the lactam unit and the exocyclic double bond) belongs to a structural architecture of Knoevenagel condensation reaction, and the compound is easily formed (Scheme 1) by the reaction of oxindole (**2**) with an appropriate pyrrole aldehyde (**3**).

In our continuing interest in kinase library generation,[11] a strategy to generate a novel class of VEGFR-2 kinase inhibitors was to build in chemical diversity around the key lactam-enone moiety of **1**. Our aim was: (1) to replace the oxindole moiety with another cyclic lactam (Step 1, Scheme 1) a critical part of our design, (2) to preserve the 3,5-dimethyl pyrrole segment, a pharmacophore presentation similar to **1**, and (3) select an oxindole replacement and expand the scope of this strategy by substituting the 3,5- dimethyl pyrrole segment with various aryl aldehydes as the condensation component in a parallel synthesis fashion (Step 2, Scheme 1).

Commercially available or known active methylene compounds (**4**–**9**) bearing a cyclic lactam unit were subjected to Knoevenagel condensation with 3,5-dimethyl pyrrole-2-carboxaldehyde (**3**) and the resulting products were tested against VEGFR-2 kinase to generate a possible hit (Fig. 1). After screening a set of compounds, we were able to generate our first hit (**10**) with a 2.7 μM inhibitory activity,[12a] which came from a condensation involving 3-phenyl-substituted pyrazolone as a single geometrical isomer.[13] We further optimized

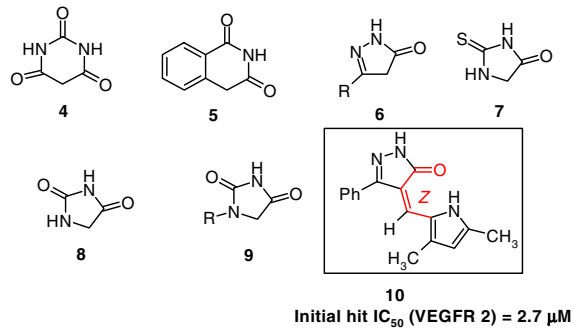
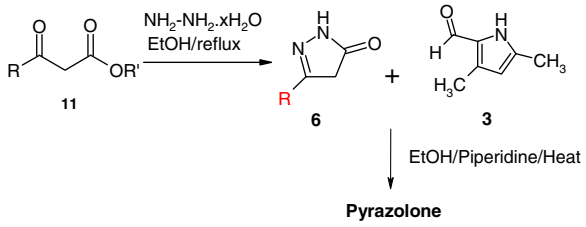


Figure 1.

the 3-substituent of our lead structure **10**, by using differently substituted pyrazolones.

A variety of pyrazolones (**6**) could be generated from the reaction of β-ketoesters (**11**) and hydrazine hydrate.[14] These were subsequently condensed with 3,5-dimethyl pyrrole carboxaldehyde (**3**) in ethanol and using piperidine as the catalyst (Scheme 2). This method of parallel library generation in solution was found favorable, as a single geometrical isomer of the double bond precipitated out of the reaction mixture in most cases and simple filtration provided compounds with high purity. We were pleased to find that the 3-substituent on the pyrazolones plays a dramatic role in enhancing potency (Fig. 2). In general, it was found that pyrazolone with an attached heterocyclic ring is preferred, while two hetero atoms on the heterocyclic ring demonstrate greater VEGFR-2 kinase inhibition.



Scheme 2.

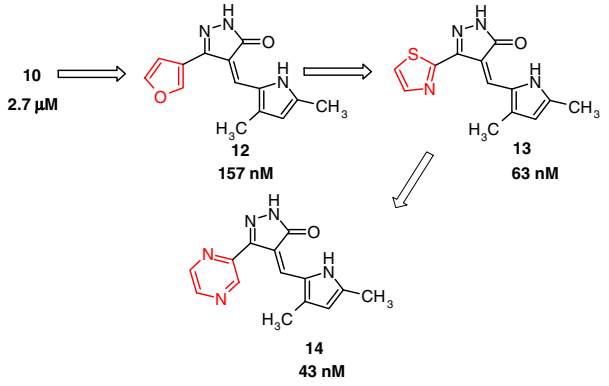


Figure 2.

After successful oxindole replacements were in hand, we turned our attention to the 2,5 dimethyl pyrrole segment of the molecules. Commercially available aryl carboxaldehydes were condensed with a series of hetero-substituted pyrazolones. After determining VEGFR-2 kinase inhibition, we found that *N*-methyindole-3-carboxaldehyde (**15**) was a potential replacement for our pyrrole, as the corresponding condensation products (data shown for **16** and **18**, Fig. 3) have comparable potencies (**14** vs **16** and **13** vs **18**).[15] Reduced kinase inhibitory activities of the compounds **17** and **19**, where the lactam NH is arylated and the exocyclic double bond has been saturated, respectively (Fig. 3), provide evidences regarding the importance of the lactam-enone pharmacophore of the pyrazolones.[10]

Structure–activity relationship (SAR) for analogs of compounds **16** and **18**, substitution on the indole ring, showed further potency enhancement against VEGFR-2 kinase, Table 1 recording representative examples. A variety of substituents are tolerated on the benzene ring of the indole nucleus, particularly at the C-4 position, resulting in several molecules with potency <10 nM. In another set of SAR, it was found that *N*-methyl group on the indole segment is favored for VEGFR-2 activity.[16]

In general, pyrazolone-based compounds demonstrated better inhibition of human VEGFR-2 in human umbilical vein endothelial (HUVEC) cell-based phosphorylation assays than against murine VEGFR-2 (FLK-1) in SVR

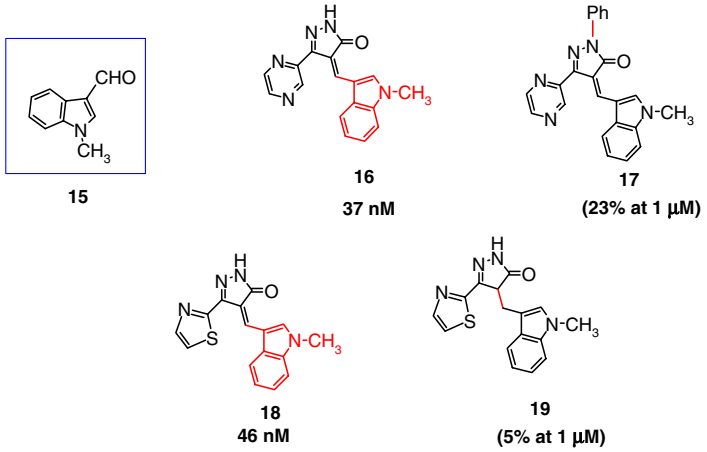
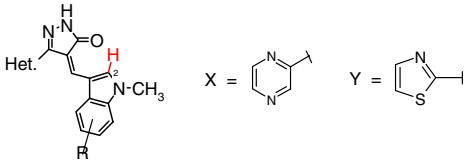


Figure 3.

Table 1. Representative examples of SAR of pyrazolones on the benzene ring of the indole segment



Compound[a]	Het.	R	IC_{50}[b] (nM)	Cell score[c]	
				HUVEC	SVR
16	X	H	37	—	—
16a	X	5-F	23	4	1
16b	X	5-Cl	10	4	1
16c	X	5-OMe	30	4	1
16d	X	4-F	17	4	1
16e	X	4-Cl	9	4	4
16f	X	4-Br	6	4	4
16g	X	4-OMe	26	4	4
16h	X	5,6-O–CH_2–O-	17	4	4
18	Y	H	46	4	0
18a	Y	5-F	49	1	0
18b	Y	5-Cl	59	—	0
18c	Y	4-Br	45	1	0
18d	Y	5,6-O–CH_2–O-	32	4	2

[a] A single geometrical isomer was isolated in each case. See Ref. 17.
[b] Ref. 12a.
[c] Ref. 12b.

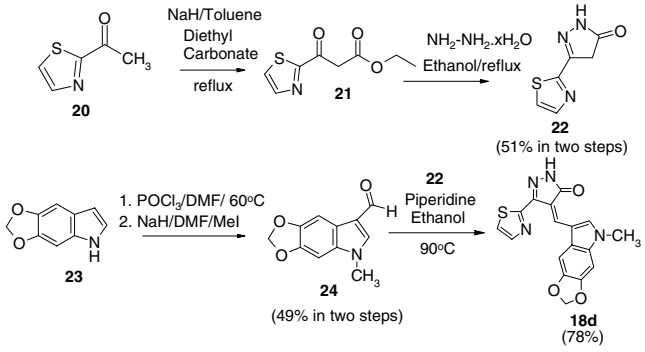
cell-based phosphorylation assays. The data for HUVEC versus SVR (SVEN 1 ras, murine endothelial pancreatic islet cells) are comparable for the inhibitors bearing 4-methoxy and halogen substituents on the indole ring of the pyrazine-based inhibitors (**16e–g**, Table 1). The cell activities for methylenedioxy group at 4 and 5 positions were also found to be good (**16h** and **18d**, Table 1).

A number of these compounds have shown significant anti-angiogenic activity ex vivo in rat aortic ring explant cultures,[18] inhibition of HUVEC capillary tube formation[19] in vitro (data not shown), and significant anti-tumor efficacy following oral administration to nude mice bearing SVR murine angiosarcoma xenografts.[20] Data have been shown for a representative compound **18d**, which was easily synthesized from the reaction of the pyrazolone **22** with the indole aldehyde **24** by heating in ethanol in the presence of piperidine (Scheme 3).

Selectivity profile against a representative panel of kinases for **18d** is shown in Table 2. While poor enzymatic inhibitions for **18d** were observed against PKC, Trk A, and CDK1/Cyclin B1, significant activities were observed against other VEGFR isozymes.

The inhibitor **18d** showed dose-related inhibition in microvessel growth relative to untreated control during peak phase of vessel growth, with 55%, 66% ($p < 0.01$), 75% ($p < 0.01$), and 90% ($p < 0.001$) inhibition observed at 4, 40, 100, and 400 nM, respectively (Fig. 4), clearly indicating anti-angiogenic properties associated with pyrazolone-based VEGFR-2 kinase inhibitors.

More importantly, compound **18d** was also tested for anti-tumor efficacy on the growth of SVR tumors (Fig. 5) in nude mice. The compound **18d** was dosed orally at 30 mg/kg twice a day for 10 days. Significant inhibition in tumor growth was noticed beginning at



Scheme 3. Synthesis of **18d**, a compound selected for anti-tumor and anti-angiogenic evaluation.

Table 2. Kinase selectivity profile for **18d**

Kinase assay	% Inhibition (IC_{50} in nM)
PKC	13% at 1 μM
Trk A	44% at 1 μM
Cdk 1/Cyclin B1	(>10,0000)
VEGFR-1	25% at 300 nM
VEGFR-3	63% at 300 nM

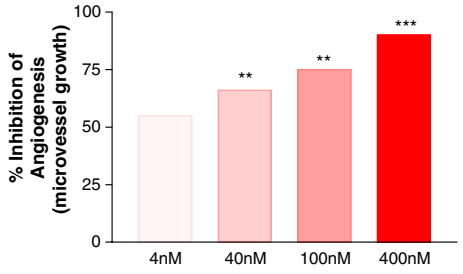


Figure 4. Dose-related effects of the pyrazolone VEGFR-2 kinase inhibitor **18d** on angiogenesis (microvessel sprouting) in serum-free collagen gel cultures of rat aortic ring explants maintained in serum-free MCDB 131 medium in the absence of exogenous VEGF. Values are the percent inhibition of microvessel growth during the peak phase of growth (day 8) relative to untreated controls; $n = 8$ replicates/concentration. Inter-assay variability <10%. $^{**}p < 0.01$ relative to untreated controls by the Student Newman–Keuls test.

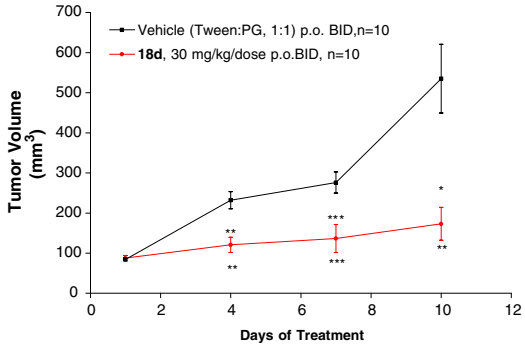


Figure 5. Effects of VEGF-R2 kinase inhibitor, **18d** on the growth of SVR murine angiosarcoma xenografts in nude mice.

day four of dosing and extending throughout the study relative to vehicle treated controls (Fig. 5).[21]

In conclusion, we have demonstrated a structure-guided library building process in solution phase, where we have grafted key structural elements of a known kinase inhibitor on different structural classes which resulted in the identification and optimization of a novel class of VEGFR-2 kinase inhibitors. SAR on pyrazolone class of molecules resulted in inhibitors which were extremely potent against the isolated enzyme as well as in cells. Some of these molecules demonstrated anti-angiogenic activity ex vivo in rat aortic ring explant cultures and oral anti-tumor efficacy in nude mice (shown for compound **18d**).

Acknowledgments

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13. Configuration of the double bond was assigned to be Z based on Sugen compounds. See Sun, L.; Tran, N.; Tang, F.; App, H.; Hirth, P.; McMahon, G.; Tang, C. *J. Med. Chem.* **1998**, *41*, 2588, for a detailed discussion on the hydrogen bonding-controlled *Z* configuration for the Knoevenagel condensation products of 2-pyrrole aldehydes.
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16. The compounds of the type **18** where the methyl group on the indole nitrogen is replaced with cyanoethyl, benzyl, benzyloxyethyl, and isobutyl groups, respectively, showed much more diminished VEGFR-2 kinase inhibitory activities (43%, 51%, 3%, and 15% inhibition at 1 μM, respectively, in contrast to the IC_{50} value of 46 nM for the compound **18**).
17. The configuration of the double bond was determined to be '*Z*' on the basis of ^{1}H NMR observations. The indole proton at 2-position (Table 1) showed a significant downfield shift (>1δ) being proximal to the lactam carbonyl in a *Z* configuration. Moreover, *Z* configuration was further confirmed from the crystal structure a pyrazolone adduct (unpublished results).
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21. Murine SVR endothelial cells (1×10^6) were injected into the right flank of the female athymic nude mice. At day 5 post-implantation when palpable tumors were confirmed, mice were randomized into treatment groups (n = 8–10) and administered **18d** at the specified concentration, orally, twice a day. Statistical analyses were done using the Mann–Whitney Sum test with $*p \leqslant 0.05$, $**p \leqslant 0.005$, and $***p \leqslant 0.001$ relative to vehicle controls. A denotes ** for **18d** at 30 mg/kg and *** for at 50 mg/kg.