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Design, synthesis, and biological evaluation of novel 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinone derivatives as potent receptor tyrosine kinase inhibitors

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ABSTRACT

A novel series of 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinone derivatives targeting VEGFR-2, PDGFR-β and c-Kit kinases were designed and synthesized. The molecular design was based on the SAR features of indolin-2-ones as kinase inhibitors. SAR study of the series allowed us to identify compounds possessing more potent inhibitory activities against the three kinases than sunitinb with IC_{50} values in the low nanomolar range in vitro. Additionally, some compounds also showed favorable antiproliferative activities against a panel of cancer cell lines (BXPC-3, T24, BGC, HEPG2 and HT29).

Over the past 15 years, protein kinases have become the most important drug targets in the field of anti-tumor drugs. So far, more than 20 kinase-targeted drugs have been used in clinical treatment, and hundreds of kinase-targeted candidates are in clinical trials.[1] Receptor tyrosine kinases (RTKs) play important roles in intracellular signal transduction. RTKs modulate cellular responses to external stimuli and influence a series of biological processes of cells, such as cell cycle, migration, metabolism, survival, proliferation and differentiation, etc.[2] Normally, signal transduction of RTKs is strictly regulated.[3] However, abnormal expression or activation of RTKs, especially excessive activations of the VEGF/VEGFR (vascular endothelial growth factor receptor) signaling pathway,[4] PDGF/PDGFR (platelet derived growth factor receptor) signaling pathway[5,6] and SCF/Kit signaling pathway,[7] will lead to series of tumor-related events, such as carcinogenesis, tumor angiogenesis, cancer metastasis, as well as disease progression and poor prognosis.[3,8] As cancer is caused by an accumulation of mutations in different genes, its growth and survival depend on more than one receptor or one signaling pathway. So theoretically multi-targeting drugs might show better curative effect than highly selective single-targeting agents. Furthermore, using a multi-targeting drug can avoid the issue of drug-drug interactions which is often a concern when combinations of single-targeting drugs are used. In fact, developing multi-targeting drugs has been considered to be a promising strategy in cancer therapy,[9,10] of which some VEGFR inhibitors have been developed in recent years, such as Sorafenib[11], Sunitinib[12], Axitinib[13] and Regorafenib[14] (Fig. 1).

Among numerous ATP-competitive VEGFR inhibitors[15,16], the scaffold of Sunitinib, 3-[(substituted pyrrol-2-yl)methylidenyl]indolin-2-one has been extensively studied and the structure–activity relationships (SAR) are described clearly based on lots of indolin-2-one derivatives (Fig. 2).[17–24] All these compounds **5**–**13** keep the similar structural features: Firstly, the amide fragments of indolin-2-ones, as the key pharmacophore, provide critical hydrogen bond acceptor and donor to interact with the amino acid residues of the kinase hinge region and remain unsubstituted in all structures. Secondly, the double bond outside indolin-2-one ring retains *cis*-configuration. Moreover, the intramolecular hydrogen bond is formed between oxygen atom of the indolin-2-one and NH of the pyrrole ring to stabilize the planar conformation of whole molecule.[16,25] Furthermore, three-dimensional model of SU5402 at the binding site of FGFR shows a highly conservative phenylalanine residue (Phe489) in the loop extends toward the indolin-2-one moiety, capping the hydrophobic pocket in which the indolin-2-one anchors.[26] There is enough space available to accommodate a larger group between exocyclic methylene and Phe489. Recently, Kim et al.,[24] reported a series of indolin-2-one structures with methylene substituted by phenyl, among which compound **13** showed very strong inhibitory activities against VEGFR-2 (IC_{50} = 10 nM), FGFR-1 (IC_{50} = 11 nM) and PDGFR-α

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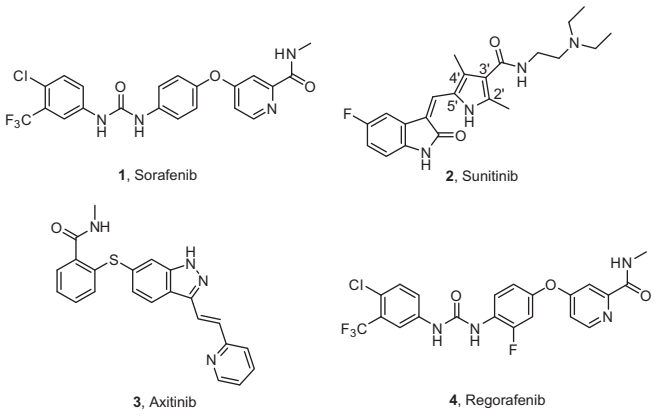


Figure 1. Approved multi-targeted VEGFR inhibitors.

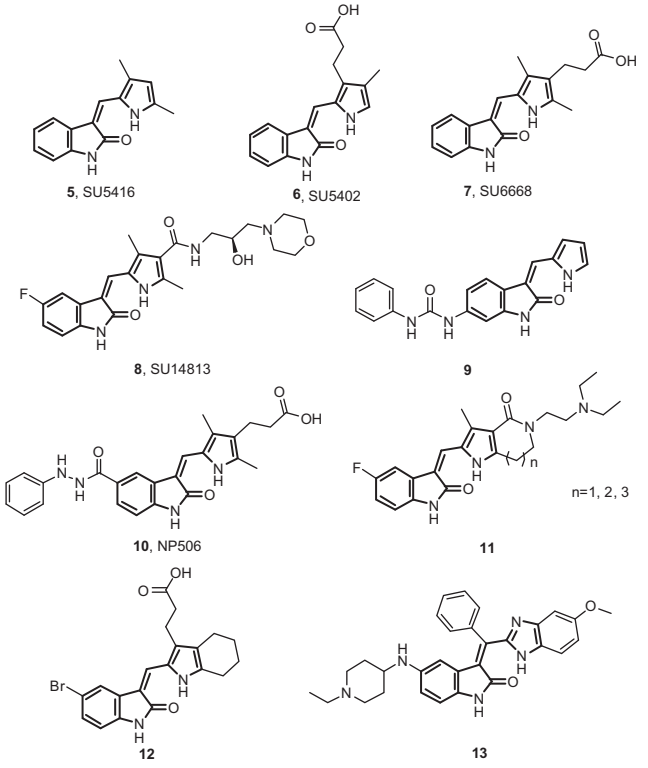


Figure 2. Representative of 3-[(substituted pyrrol-2-yl)methylidenyl]indolin-2-ones.

(IC_{50} = 6 nM). This further confirms that hydrophobic groups are tolerated at the region between the 4′ position of pyrrole ring and exocyclic methylene of indolin-2-one.

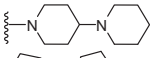
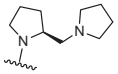
Since the ATP-binding domain of FGFR, VEGFR-2 and PDGFR is highly homological and conservative, the binding mode and SAR based on FGFR can also be applied to other kinases.[18] We designed and synthesized a novel class of 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinone derivatives **21**–**44** (Table 1) according to the known SARs and binding information. The inhibitory activities against VEGFR-2, PDGFR-β and c-Kit were then tested. In the new scaffold, indolin-2-one as the key pharmacophore was retained and a six member ring was formed by connecting the methyl group at 4′ position of the pyrrole ring and the double bond outside of the indolinone via two methylenes. Meanwhile, the intramolecular hydrogen bond between oxygen atom of the indolin-2-one and NH of the pyrrole ring was kept to stabilize the planar conformation similar to sunitinib and it's analogs **5**–**13**. In addition, free rotation of the single bond between the exocyclic double bond and 5′ position of the pyrrole ring was further restricted by introducing a rigid six-membered ring which would help to make pharmacophoric conformation more stable. Our primary objective was to maintain the planar conformation of the molecule by introducing a rigid ring structure while retaining the key intramolecular hydrogen bond, and improve the biological activities of these novel scaffold compounds as kinase inhibitors. Additionally, this study helped to expand the structural diversity of indolin-2-ones as kinase inhibitors and establish more specific structure–activity relationships.

Compound **18** is a key intermediate in building 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinones scaffold. Herein, 6-amino-5-oxohexanoic acid hydrochloride[27], as the starting material, was cyclized with ethyl acetoacetate to form pyrrole ring **16**, which underwent intramolecular dehydration to give compound **17** in the presence of P_2O_5. Hydrolysis of compound **17** under alkaline condition produced 2-methyl-7-oxo-4,5,6,7-tetrahydro-1*H*-indole-3-carboxylic acid **18**. The substituted indolin-2-ones **19** was either commercially available or easy to be prepared according to the procedures described in literature.[28,29] The final products were synthesized through the following steps: firstly, the condensation of substituted indolin-2-ones with compound **18** was catalyzed by $TiCl_4$ in pyridine to generate intermediate **20**; which reacted with primary or secondary amines in the presence of coupling reagent EDCI/HOBt (Scheme 1) to afford the final products **21**–**44**. In addition, to verify the configuration of the exocyclic double bond of the oxyindole ring, The two dimensional (2D) NMR spectroscopy of compound **22** was conducted which showed a ROESY correlation between proton 6′ and proton 4. Accordingly, the double bond was assigned as configuration *Z*. This configuration was further confirmed by single-crystal X-ray diffraction of compound **22** methanol solvate as shown in Figure 3.[30]

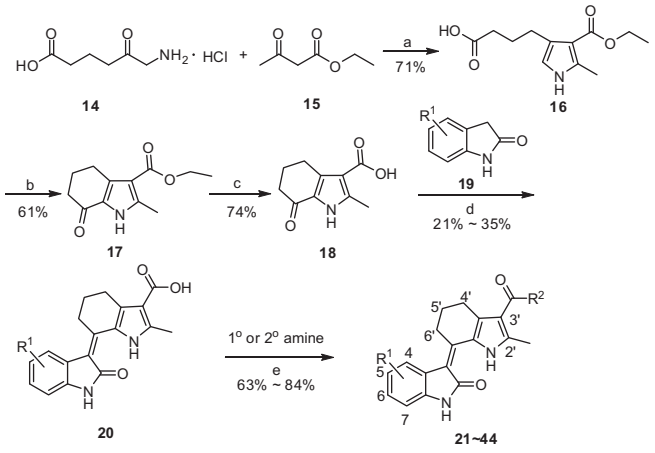
The in vitro inhibitory activities against VEGFR-2, PDFR-β and c-Kit of the synthesized compounds were shown in Table 1. Most of 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinone derivatives exhibited potent inhibitory activities. Herein, compound **22** exhibited better inhibitory activities against VEGFR-2, PDGFR-β and c-Kit than the reference compound, sunitinib, which indicated that our design was reasonable. To further investigate the influence of different substituents at the scaffold and gain insight into SAR for this series of molecules, we firstly kept the R^2 as *N*,*N*-diethyl ethylenediamine and modified the substituents of indolin-2-ones moiety. A total of 11 compounds **21**–**31** were prepared, whose inhibitory activities against the VEGFR-2, PDGFR-β and c-Kit were tested with IC_{50} values in the range of 2.3–330.3 nM, 7.6–395.7 nM and 2.3–397.6 nM, respectively. The results indicated that changing the substituents on indolin-2-ones would significantly affect the inhibitory activity of the compounds.

Introducing fluorine atom to the 4 or 7 position of the indolin-2-ones moiety led to significant decrease of the inhibitory activities to VEGFR-2, PDGFR-β and c-Kit. For example, the inhibitory activity of compound **31** to all three kinases decreased significantly, with IC_{50} all greater than 100 nM. Moreover, since the indolin-2-one moiety stretched deeply into the ATP-binding pocket, the introduction of large substituents at 5 position, such as methyl carboxylic ester **28** and sulfonyl diethylamine **29**, would hinder the binding of the ligand to receptor sterically and significantly reduce the inhibitory activity. On the other hand, the compounds with smaller substituents at the 5 position of indolin-2-ones, such as fluorine **22**, chlorine **23**, bromine **24** and methyl **25**, exhibited better inhibitory activities against VEGFR-2 and c-Kit than the compound without any substitutions **21**, and different halogen substitutions had little effect on the potency.

Table 1
IC_{50} values (nM) of kinase inhibition for compounds **21**–**44** and Sunitinib

Compd	R^1	R^2	Biochemical activity (IC_{50}, nM)[a]		
			VEGFR-2	PDGFR-β	c-Kit
21	H	$NH(CH_2)_2N(CH_2CH_3)_2$	9.4	89.9	
22	5-F	$NH(CH_2)_2N(CH_2CH_3)_2$	2.3	10.1	2.4
23	5-Cl	$NH(CH_2)_2N(CH_2CH_3)_2$	2.6	12.1	2.7
24	5-Br	$NH(CH_2)_2N(CH_2CH_3)_2$	3.9	7.6	2.3
25	5-CH_3	$NH(CH_2)_2N(CH_2CH_3)_2$	2.3	15.6	4.1
26	5-OCH_3	$NH(CH_2)_2N(CH_2CH_3)_2$	24.6	101.4	35.5
27	5-NO_2	$NH(CH_2)_2N(CH_2CH_3)_2$	25.7	13.3	6.9
28	5-$COOCH_3$	$NH(CH_2)_2N(CH_2CH_3)_2$	167.2	70.3	255.7
29	5-$SO_2N(CH_2CH_3)_2$	$NH(CH_2)_2N(CH_2CH_3)_2$	330.3	395.7	397.6
30	4-F	$NH(CH_2)_2N(CH_2CH_3)_2$	15.0	62.1	43.1
31	7-F	$NH(CH_2)_2N(CH_2CH_3)_2$	112.4	260.3	180.8
32	5-F	$NH(CH_2)_2$-morpholin-4-yl	1.6	13.2	2.0
33	5-F	$NH(CH_2)_2$-piperidin-1-yl	4.3	19.4	5.8
34	5-F	$NH(CH_2)_2$-pyrrolidin-1-yl	9.7	41.6	7.4
35	5-F	$NH(CH_2)_2N(CH_3)_2$	3.0	22.7	6.6
36	5-F	$NH(CH_2)_2OH$	1.1	12.7	1.4
37	5-F	$NH(CH_2)_2$-pyridin-2-yl	11.4	160.6	37.7
38	5-F	$NH(CH_2)_3N(CH_2CH_3)_2$	2.7	21.3	9.9
39	5-F	$NH(CH_2)_3$-morpholin-4-yl	1.0	18.0	5.7
40	5-F	$NH(CH_2)_3$-pyrrolidin-1-yl	3.8	64.8	10.7
41	5-F	$NHCH_2CH(OH)CH_2N(CH_2CH_3)_2$	2.5	19.3	4.1
42	5-F	Morpholin-4-yl	1.1	40.1	2.1
43	5-F		8.0	40.7	7.8
44	5-F		11.8	118.4	8.5
Sunitinib [b]	—	—	4.0	10.6	8.9

[a] IC_{50} values were determined by at least two separate tests with deviation less than 20% and are reported as mean values.
[b] IC_{50} value obtained under our experimental conditions.



Scheme 1. Reagents and conditions: (a) NaH_2PO_4, H_2O, reflux, 0.5 h; (b) P_2O_5, PPA, 70 °C, 48 h; (c) LiOH, 80 °C, 24 h; (d) $TiCl_4$, Py, 90 °C, 12 h; (e) EDCI, HOBt, DMF, rt, 24 h.

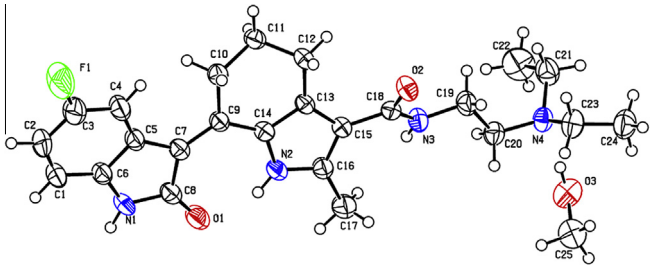


Figure 3. Molecular structure of compound **22** methanol solvate.

In addition, we prepared 13 compounds (**22**, **32**–**44**) with 5 position of indolin-2-ones fixed as fluorine to explore the influence of different side-chain substituents at 3′ position. Their inhibitory activities against the VEGFR-2, PDGFR-β, and c-Kit were tested and their ranges of IC_{50} were 1–11.8 nM, 10.1–160.6 nM and 1.4–37.7 nM, respectively, which suggested that changes of substituents at the side chain had slight effect on the activity against VEGFR-2 but had significant effect on the activities against PDGFR-β and c-Kit. Especially, when R^2 was $NH(CH_2)_2$-pyridin-2-yl 37, the inhibition against PDGFR-β and c-Kit decreased notably, with IC_{50} at 160.6 nM and 37.7 nM, respectively.

To understand how these multi-targeted kinase inhibitors interact with the receptor, the compound **22** and VEGFR-2 (PDB: 3C7Q[31]) was submitted for docking studies using Surflex-dock in SYBYL7.3. The results revealed that compound **22** bound to the ATP-binding site in the cleft between the NH_2 and COOH terminal lobes of the kinase domain and kept the same molecular orientation as original ligand BIBF 1120 (Fig. 4a). The indolinone moiety stretched into the receptor-binding pocket deeply, while the side chain oriented towards outside. The indolinone scaffold formed

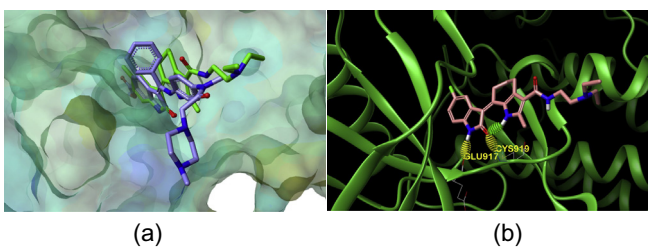


Figure 4. Compound **22** and BIBF 1120 in the ATP binding pocket of VEGFR-2 (PDB entry 3C7Q). (a) Compound **22** (green) was overlapped with BIBF 1120 (cyan) in binding pocket. (b) The interaction between compound **22** and VEGFR-2. The picture is prepared in Benchware 3D Explorer 2.3 (Tripos, St. Louis, MO).

Table 2
Antiproliferative activity of the target compounds and Sunitinib against selected cancer cell lines

Compd	Cell lines IC_{50} (μM)				
	BXPC-3	T24	BGC	HEPG2	HT29
21	0.52	3.56	6.73	7.73	0.54
22	0.52	3.31	6.11	7.27	1.2
23	1.95	1.83	2.03	3.14	6.48
25	2.43	NT[b]	3.15	NT	1.73
Sunitinib [a]	3.63	2.44	4.78	5.61	1.47

[a] IC_{50} value obtained under our experimental conditions.
[b] Not tested.

two hydrogen bonds with the backbone nitrogen of Cys919 and the backbone carbonyl oxygen of Glu917 in the hinge region. A similar intramolecular hydrogen bond was also formed between the NH-1′ and carbonyl at the C-2 position of the indolin-2-one just like compounds **2** and **5**–**13** (Fig. 4b).

Moreover, some of the newly synthesized indolin-2-ones (**21**, **22**, **23**, **25**) were evaluated for in vitro antiproliferative activity against five human cancer cell lines, including BXPC-3 (primary pancreatic adenocarcinoma), T24 (bladder carcinoma), BGC (Gastric carcinoma), HEPG2 (liver hepatocellular carcinoma) and HT29 (Colon Carcinoma) using a standard MTT assay (Table 2).[32] They exhibited potent anti-proliferative activities against all five cell lines with IC_{50} values in the range of 0.52–7.73 μM, and especially more effective than the reference drug in inhibiting the growth of the BXPC-3 cell lines. In particular, compound 21 (IC_{50} = 0.52 μM) and 22 (IC_{50} = 0.52 μM) were found to be sevenfold more potent than Sunitinib in BXPC-3.

Furthermore, compound 22 had similar kinase selectivity as sunitinib. In addition to VEGFR-2, PDGFR-β and c-Kit, it also had strong inhibitory activities against VEGFR-1, VEGFR-3, Ret and Flt-3, with IC_{50} ranging from 1.3 to 9.2 nM. PK studies in rats, dogs and cynomolgus monkeys revealed that compound 22 had higher oral bioavailability (~90%) in cynomolgus monkeys than in rats or dogs. Compound **22** also showed strong efficacy in multiple human xenograft mouse tumor models, including HT-29, A549, MDA-MB-231, SK-OV-3, SW-620, Bel-7402, BGC-823, HePG2 and NCI-H526.

In summary, we reported herein the design and synthesis of novel 3-pyrrolo[*b*]cyclohexylene-2-dihydroindolinone derivatives based on the SAR features of indolin-2-ones as kinase inhibitors. Most of the newly synthesized compounds exhibited potent inhibitory activities against VEGFR-2, PDGFR-β and c-Kit in biochemical assays. Initial SAR study suggested that structural modifications at phenyl ring of indolin-2-ones had a dramatic effect on the potency of these agents. Furthermore, molecular docking study indicated that indolin-2-one moiety of compound **22** formed two key hydrogen bonds with the backbone nitrogen of Cys919 and the backbone carbonyl oxygen of Glu917 in the ATP binding site of VEGFR-2. Additionally, some compounds also showed favorable antiproliferative activity against a panel of cancer cell lines (BXPC-3, T24, BGC, HEPG2 and HT29). Further PK and in vivo efficacy studies showed that compound **22** was potentially an ideal drug candidate for cancer treatment.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.037.

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