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Original article

Synthesis and biological evaluation of 2-indolinone derivatives as potential antitumor agents

Hongbin Zou [a], Liang Zhang [a], Jingfeng Ouyang [a], Marc A. Giulianotti [b], Yongping Yu [a,b,*]

[a] College of Pharmaceutical Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, PR China
[b] Torrey Pines Institute for Molecular Studies, 11350 Village Parkway, Port St. Lucie, FL 34987, USA

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ABSTRACT

Three series of 3-substituted-indolin-2-ones and azaindolin-2-ones have been synthesized and showed potential antiproliferative activity to cancer cell lines. The inhibition activities on VEGF-induced VEGFR phosphorylation were observed for selected 2-indolinones. Among the compounds synthesized, 5-fluoroindolin-2-one derivative **23** with a pyridone unit showed the most significant enzymatic and cellular activities. Flow cytometric analysis indicates that **23** plays a role in suppressing HCT-116 cell proliferation via G1 phase arrest and apoptosis in a dose dependent manner. The binding mode of compound **23** complexed with VEGFR-2 was predicted using FlexX algorithm. Described here are the chemistry and biological testing for these series which will guide the design and optimization of novel 2-indolione antitumor agents.

1. Introduction

Oxindole and its derivatives are frequently occurring structure units in natural products and pharmacologically important compounds that display utilizations in many major therapeutic areas [1–4]. Among them, SU5416 (**1**) possessing a 3-methyleneindolin-2-one substructure, was found to be a tyrosine kinase inhibitor targeting vascular endothelial growth factor receptor (VEGFR) [5]. In the search for novel and potent anticancer agents, studies involving 3-methyleneindolin-2-ones have been reported (**2**–**4**, Fig. 1) [6–12]. Among them, SU11248 (Sunitinib, **3**) was approved by the FDA for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumors (GIST) [13,14]. Preliminary evidence has also suggested promising activity of SU11248 in other tumor groups and showed improvement in the therapeutic efficacy of advanced solid tumors by combination SU11248 with other chemotherapeutic agents [15–17]. However, with long time exposure to Sunitinib, adverse effects have been observed during treatment such as arterial hypertension, fatigue, oral disorders and diarrhea [18].

Recently Cho found pyrrolo-fused six-, seven-, and eight-member heterocycle derivatives in which the novel oxindole derivative **4** was found to be orally active, showing better in vivo antitumor activity in comparison with SU11248 (**3**) [12]. Herein we reported the synthesis and biological evaluation of 5-fluorooxindoles and 4-, 5-, 7-azaoxindole derivatives **12**–**36** (Scheme 1). The oxindole pharmacophore was maintained and nitrogen was introduced into the skeleton (**9**–**11**, Scheme 1) in order to improve the water solubility and unique hydrogen bonding properties which were also seen for the indole scaffold [19,20]. To obtain an insight into the effect of the 3-substituents of oxindole on the biological activities of these compounds, the substituted pyrrole-2-carbaldehydes, pyridone-2-carbaldehydes and indole-3-aldehydes were chosen for condensation with the various 2-indolinone units (**8**–**11**, Scheme 1). All the synthesized compounds (**12**–**36**, Scheme 1) were used to screen six cultured human cancer cell lines (PC-3, K562, HL60, HCT-116, A549 and HepG2) and one human normal cell line QSG-7701. The results showed that the compounds have cytotoxic potential and compound **23** exhibited the most significant cytotoxicity against these cancer cell lines, especially against HCT-116. Further biological analyses and molecular modeling on selected compounds were carried out to determine the possible mechanism involved in the cytotoxicities.

2. Chemistry

The targeted compounds (**12**–**36**, Table 1) were obtained by condensation of substituted aldehydes (**5**–**7**, Scheme 1) with

* Corresponding author. Institute of Material Medica, College of Pharmaceutical Sciences, Zhejiang University, 383 Yu Hang Tang Road, Hangzhou 310058, PR China. Tel./fax: +86 571 88208452.
E-mail address: yyu@zju.edu.cn (Y. Yu).

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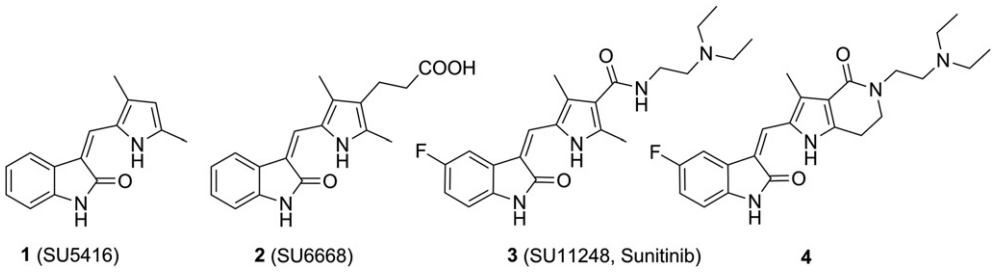


Fig. 1. Developed antitumor 3-methyleneindolin-2-ones.

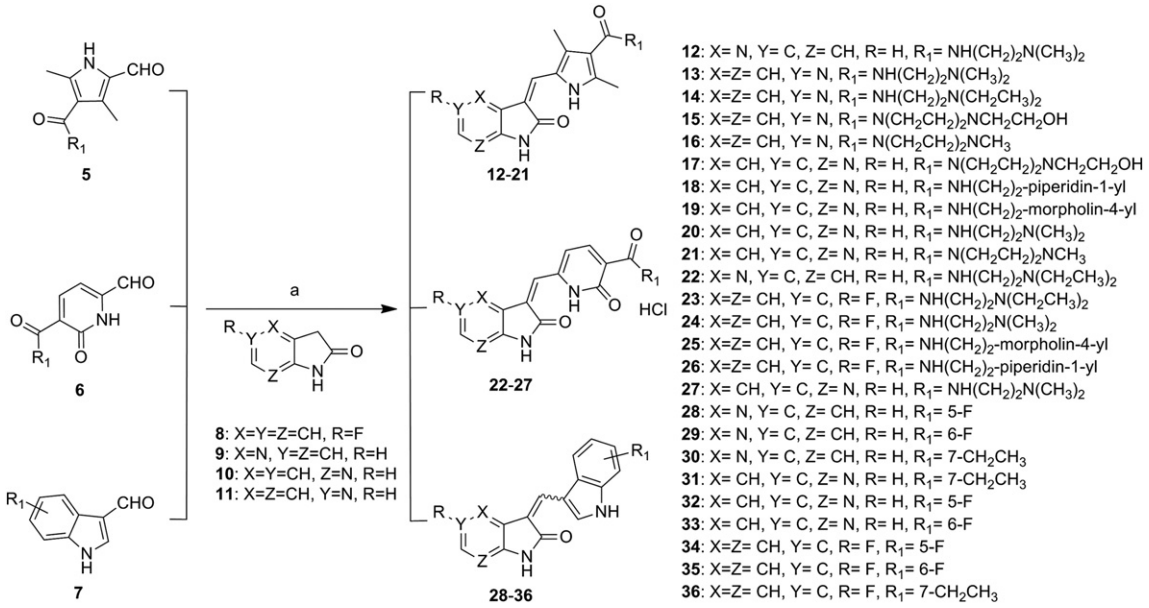
various 2-indolinone derivatives (**8**–**11**, Scheme 1) in the presence of piperidine as shown in Scheme 1 [6]. Among them, the hydrochloride derivatives **22**–**27**, which have good water solubility, were synthesized by additional stirring in the presence of hydrogen chloride saturated methanol. The substituted pyrrole-2-aldehydes (**5**) and pyridone-2-carbaldehydes (**6**) were prepared by amidation of the corresponding **5a** and **6a** with different aimines respectively (Scheme 2). The pyrrole-3-carboxylic acid (**5a**) and the pyridone-3-carboxylic acid (**6a**) were synthesized utilizing reported conditions [6,21]. The indole-3-aldehydes (**7**) were prepared from substituted indoles as previously described with the yields over 85% [22]. The 5-fluoroindolin-2-one (**8**) was commercially available while the 4-azaindolin-2-one (**9**) was synthesized from 2-chloro-3-nitropyridine in the same way that was reported by Finch [23]. Bromination of 5-azaindole (**10a**) and the following Pd–C catalyzed reduction afforded the 5-azaindolin-2-one (**10**, Scheme 2) in which **10a** was prepared according to the reported method [24,25]. A similar approach was utilized to obtain 7-azaindolin-2-one (**11**, Scheme 2) from the commercially available 7-azaindole (**11a**).

The configurations of the isolated compounds were confirmed by mass spectrometry and NMR spectra. Because of the exocyclic double bond, the oxindole derivatives may exist as the *E* or *Z* isomer or as mixture of both. For the synthesized oxindole derivatives with either a pyrrole or a pyridone moiety (**12**–**27**) a single *Z* configuration was obtained due to the intramolecular interaction (**23** as an example, Fig. 2) [26,27]. The results also showed the existence of the inseparable *E*/*Z* mixtures for compounds **28**–**30** and the *E*/*Z* ratios were determined using the corresponding chemical shifts and integrals in the ^{1}H NMR spectra [27]. As shown in Table 1, the inseparable *E*/*Z* mixtures (**28**–**30**) contain the 4-aza-2-indolinone scaffold which suggested that both possible indicated intramolecular interactions between H-2′ and the scaffold (**29**, Fig. 2) might be the main reason for the two isomers.

3. Results and discussion

All of the synthesized oxindole derivatives were evaluated for cytotoxic activity in vitro against six human cancer cell lines (PC-3, K562, HL60, HCT-116, A549 and HepG2) and one human normal cell line QSG-7701 using SU11248 as a positive control. Results showing the concentrations required to inhibit cell growth by 50% (IC_{50} values) are presented in Table 1.

The bioassay results suggest that most of the synthesized 2-indolinones and aza-2-indolinones have cytotoxic potential to these six human cancer cell lines while only two compounds (**30**, **31**) show weak cytotoxicities to QSG-7701 cell line (Table 1). Analysis of the MTT assay results of compounds **12**–**21** with pyrrole moiety, suggests that when the R_1 substituent is a piperazine moiety (**15**–**17** and **21**, Table 1) the compounds lack cytotoxicity. Comparing compounds **12**, **13**, **20** with SU11248 (**3**), the bioassay results indicate that the aza-2-indolinone scaffold with incorporated nitrogen at



Scheme 1. Synthesis of 2-indolinone derivatives. Reagents and conditions: (a) piperidine, methanol, reflux, 6 h. For **22**–**27**, additional stirring in hydrogen chloride saturated methanol is needed. Yields 60–92% were obtained.

Table 1
Synthesized 2-indolinones **12**–**36** and their cytotoxicities on seven human cell lines.[a]

Compd	PC-3	K562	HL60	HCT-116	A549	HepG2	QSG-7701
3	25.1 ± 2.1[b]	21.9 ± 1.3	15.5 ± 1.0	6.1 ± 0.4	18.5 ± 1.1	15.4 ± 0.5	–
12	47.0 ± 5.3	24.1 ± 1.7	45.9 ± 2.3	12.4 ± 1.5	16.7 ± 2.1	20.2 ± 0.6	–
13	–[b]	15.7 ± 1.1	34.8 ± 1.6	9.1 ± 0.4	12.5 ± 0.6	8.6 ± 0.4	–
14	–	14.8 ± 0.8	35.0 ± 2.0	11.0 ± 1.8	13.9 ± 0.3	16.4 ± 0.5	–
15–**17**	–	–	–	–	–	–	–
18	25.6 ± 1.6	18.0 ± 1.2	30.2 ± 1.5	18.1 ± 1.2	25.4 ± 1.3	38.6 ± 0.7	–
19	–	–	27.8 ± 1.3	25.5 ± 1.8	29.2 ± 1.2	–	–
20	49.5 ± 3.7	19.8 ± 0.9	48.8 ± 2.2	9.2 ± 0.5	21.0 ± 0.9	18.3 ± 0.5	–
21	–	–	40.0 ± 2.5	35.0 ± 0.8	48.2 ± 2.7	37.3 ± 0.6	–
22	30.3 ± 1.5	25.3 ± 3.1	32.9 ± 2.8	8.2 ± 0.5	11.8 ± 0.7	12.6 ± 0.2	–
23	21.0 ± 1.2	13.8 ± 1.1	22.7 ± 1.7	2.3 ± 0.1	6.2 ± 0.3	5.0 ± 0.2	–
24	25.2 ± 1.1	19.3 ± 1.2	24.7 ± 1.6	5.3 ± 0.3	12.6 ± 0.2	17.1 ± 0.4	–
25	35.6 ± 1.5	45.1 ± 2.3	38.9 ± 2.3	12.8 ± 0.2	22.0 ± 0.4	35.0 ± 0.7	–
26	28.0 ± 2.4	26.8 ± 1.4	32.7 ± 1.1	9.5 ± 0.4	15.2 ± 0.3	18.9 ± 0.5	–
27	26.0 ± 2.2	21.3 ± 0.5	34.3 ± 1.8	4.3 ± 0.3	13.7 ± 0.6	27.3 ± 0.5	–
28[c]–**29**[d]	–	–	–	–	–	–	–
30[d]	–	–	34.2 ± 2.1	22.3 ± 1.1	32.5 ± 0.9	–	44.8 ± 0.5
31[e]	–	27.7 ± 1.3	37.4 ± 1.9	13.6 ± 0.8	17.1 ± 0.7	10.5 ± 0.3	42.6 ± 0.7
32[e]	–	30.0 ± 2.1	48.0 ± 2.6	17.8 ± 1.0	–	–	–
33[e]	–	–	34.1 ± 1.8	22.3 ± 1.5	31.1 ± 1.2	15.4 ± 0.4	–
34[e]	–	–	–	42.1 ± 1.6	–	–	–
35[e]	–	–	43.1 ± 2.1	28.0 ± 1.5	–	–	–
36[e]	26.4 ± 1.8	15.8 ± 0.8	26.7 ± 1.5	8.6 ± 0.8	11.0 ± 1.1	28.1 ± 0.3	–

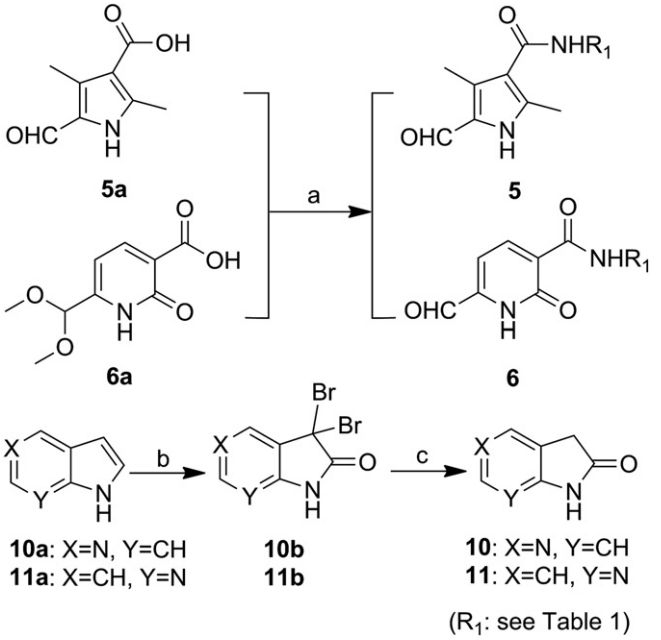
[a] Key to cell lines: PC-3, human prostate cancer cell line; K562, human erythroleukemia cells; HL60, human promyelocytic leukemia cell line; HCT-116, human colon cancer cell line; A549, human lung adenocarcinoma cell line; HepG2, *human liver carcinoma cell line;* QSG-7701, human liver cell line.
[b] IC_{50} values greater than 50 μM were considered as inactive and omitted here.
[c] Configuration ration: *Z*/*E* 5:2.
[d] Configuration ration: *Z*/*E* 1:5.
[e] *Z* configuration ration.

different positions provides no increase of the cytotoxicity to the selected cancer cell lines in vitro. Compounds **30**–**36** with an indole moiety exhibited selective cytotoxicities to HCT-116 and HL60 cancer cell lines while compound **36** showed potent cytotoxicity to all of the selected cancer cell lines, similar to that seen for **3**.

Analysis of the MTT assay results, also suggests that the HCT-116 cell line is much more sensitive to the synthesized oxindole derivatives and analogs with a pyridone moiety are generally more potent than those with pyrrole and indole moiety. Among them, compound **23** showed the most significant cytotoxicity to these cell lines, especially to the HCT-116, A549 and HepG2 cell lines, with IC_{50} values of 2.3, 6.2 and 5.0 μM, stronger than that seen for SU11248 (**3**).

To gain additional insight into the possible mechanism involved in the cytotoxicity, HCT-116 cell line treated with compound **23** was chosen for further biological analysis. The effect of compound **23** on cell cycle distribution was investigated by flow cytometric anaylsis. HCT-116 cells were treated with 12.5, 25 and 50 μM of compound **23** for 48 h which resulted in the accumulation of G1 phase, from 36.8% to 42.63%, 45.44% and 81.63%, respectively (Fig. 3A). The apoptotic effect of compound **23** was evaluated using annexin V and PI staining and a dose dependent relationship was also observed (Fig. 3B). After treatment with compound **23** for 48 h, the cell apoptotic population increased from 4.17% of the control group to 20.48%, 28.63% and 48.67% (Fig. 3B). These results suggest that compound **23** inhibited HCT-116 cell viability via G1 phase arrest and apoptosis in a dose dependent manner.

The synthesized targeted compounds **12**–**36** share the same 2-indolinone scaffold with compounds **1**–**4** which are good VEGFR-2 inhibitors [5,6,12]. To determine whether the VEGFR-2 inhibitory activity plays a rule in the observed cytotoxicity, the representative compounds **13**, **23** and **36** with potent cytotoxicities were selected for further analysis. HCT-116 cells that naturally express VEGFR-2 were chosen for the cellular inhibition assay of **13**, **23** and **36** on the VEGF-induced VEGFR phosphorylation [28,29]. Interestingly, all of them showed inhibitory potency and **23** was noted to be the most effective of the three compounds against VEGF-induced VEGFR-2 phosphorylation (Fig. 4). These compounds were further screened for the in vitro inhibition of VEGFR-2 activity and the results were shown in Table 2. All of the tested compounds showed inhibitory potency. Among them, compound **23** exhibited the most significant activity against VEGFR-2 with an enzymatic IC_{50} of 0.015 μM which is identical to that seen for Sunitinib (**3**) with an enzymatic IC_{50} of 0.012 μM.



Scheme 2. Synthesis of several key intermediates. Reagents and conditions: (a) EDC, HOBt, DMF, amines, rt, 12 h, 66% for **5**, 84% for **6**; (b) Br_2, *t*-BuOH, 20 min, 65% for **10b**, 77% for **11b**; (d) Pd/C, H_2, CH_3CH_2OH, rt, 12 h, 62% for **10**, 76% for **11**.

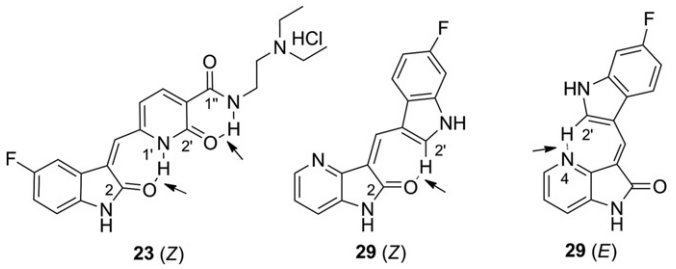


Fig. 2. The intramolecular interaction of the synthesized compounds.

To illustrate the detailed possible interaction mechanism, a potential binding model was constructed. Compound **23**, which was the compound that showed the most significant enzymatic and cellular activities, was chosen and docked into the ATP-binding site of VEGFR-2 (PDB entry 1Y6A) using FlexX program [30]. The predicted binding mode of **23** to VEGFR-2 is shown in Fig. 5, in which the indolinone scaffold forms two hydrogen bonds to the backbone amine group of residues Lsy866 and Asp1044, while the diethylamine tail group is exposed to solvent without specific contacts with VEGFR-2. The backbone carbonyl group of residue Glu883 potentially interacts through a hydrogen bond with the amine of pyridone unit.

4. Conclusions

In summary, three series of novel indolinone-based chemical entities with potential antiproliferative activities were synthesized in which the pyridone-type compounds are much more sensitive to the selected six cancer cell lines. Further bioassay results, especially involving compound **23**, show that the indolinone plays a role in suppressing HCT-116 cell proliferation via G1 phase arrest and apoptosis in a dose dependent manner. The inhibition activity of **23** on VEGFR-2 and VEGF-induced VEGFR phosphorylation were also observed which was confirmed by a predicted binding mode of **23** complexed with VEGFR-2. The results reported here provide a foundation for further improvement of the potency of these compounds to discover more potent novel indolinone antitumor agents in future studies.

5. Experimental

5.1. Materials

Melting points were measured on a Perkin-Taike X-4 apparatus and then corrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian INOVA 400 spectrometer with TMS as an internal standard and $CDCl_3$ as solvent. ESI-MS data were recorded on a Bruker Esquire 3000+ spectrometer. TLC was performed on silica gel (GF_{254}). Column chromatography was carried out on silica gel H (10–40 μm). All of the silica gel GF_{254} and silica gel H were purchased from Qingdao Marine Chemical Factory, China. The MTT assay was measured at 570 nm using a multiskan spectrum (Thermo Electron Co., Vantaa, Finland). The primary antibodies to VEGFR and p-VEGFR were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). The peroxidase-coupled secondary antibody was obtained from Southern Biotech (Birmingham, UK) and ECL-plus kit was from Amersham Biosciences (UK) for detection. The Eu-cryptate labeled anti-phosphotyrosine antibody (PY20K) was purchased from CAS Bio International. The modified APC labeled Streptavidin (SV-XL) was obtained from CIS Bio International.

5.2. Synthesis

5.2.1. General procedure for the preparation of intermediates

*5.2.1.1. General procedure for the preparation of aza-oxindolin-2-ones (**10**, **11**).* To a stirred solution of azaindole (**10a** or **10b**,

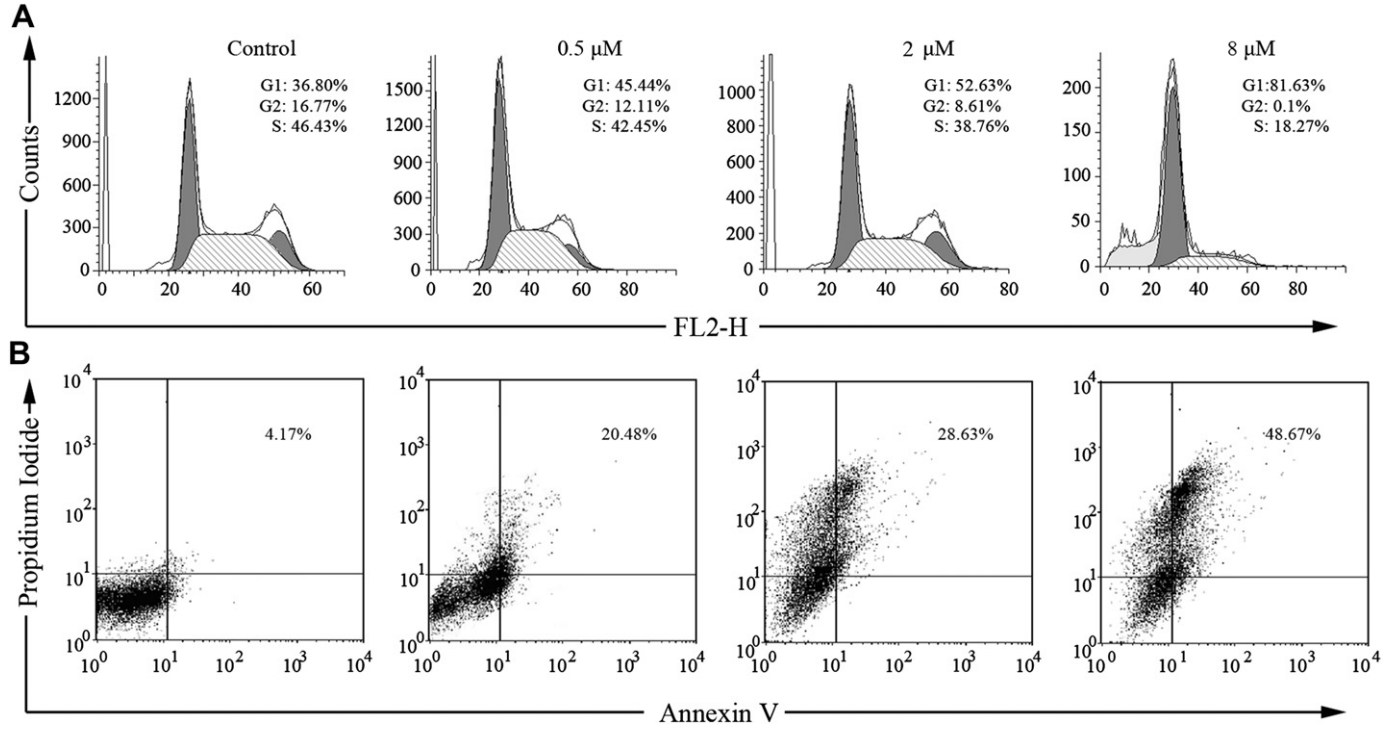


Fig. 3. Cell cycle progression (A) and apoptosis (B) involved in compound **23** cytotoxicity on HCT-116 cells. Cells were treated with **23** at indicated concentrations for 48 h. (A) Cells treated with **23** were washed and collected. The cells were stained with propidium iodide and then DNA contents were analyzed by flow cytometry. (B) Flow cytometric analysis of phosphatidylserine externalization (annexin V-binding) and cell membrane integrity (PI staining). Cell apoptosis rate in each group was marked.

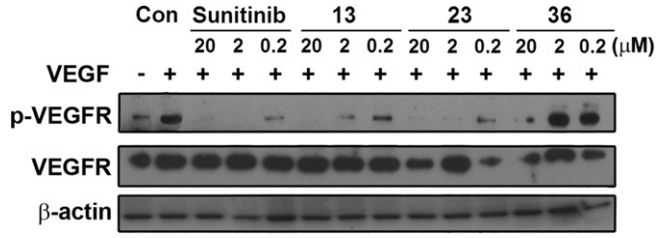


Fig. 4. Inhibition of **23** on VEGF-induced VEGFR-2 phosphorylation. HCT-116 cells were pre-incubated with indicated concentrations of compound for 30 min followed by 50 ng/mL VEGF for 2 h.

Table 2
Enzymatic activities of selected compounds toward VEGFR-2 kinase.

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)
3	0.012	**23**	0.015
13	0.060	**36**	6.58

[a] Determined against isolated VEGFR-2 for three independent experiments (at least 8 points per experiment).

12.7 mmol) in tert-butyl alcohol (100 mL) and H_2O (100 mL) at room temperature, Br_2 (2.6 mL, 50.5 mmol) was added dropwise over 20 min. The mixture was stirred for 1 h and the yellow precipitate (**10b**, or **11b**) was collected by filtration. The dried precipitate (**10b**, or **11b**, 4.8 mmol) was refluxed with 5% Pd–C (1.0 g) in 60 mL of 8.8% HCOOH/CH_3OH for 12 h under nitrogen. The catalyst was removed by filtration through a pad of celite. After removal of the solvent, the residual was adjusted to pH 8–9 by addition of saturated K_2CO_3 solution. The solution was stored in the refrigerator overnight and the precipitate was filtered to give 5-azaindolin-2-one (**10**) or 7-azaindolin-2-one (**11**).

*5.2.1.2. General procedure for the preparation of substituted aldehydes (**5**, **6**).* The substituted acid (**5a** or **6a**, 1.0 mmol), EDC (229 mg, 1.2 mmol) and HOBt (160 mg, 1.2 mmol) were dissolved in DMF (25 mL). After cooling to 5 °C, the substituted amine was added to the mixture and was stirred at room temperature overnight followed by evaporation under reduced pressure to remove DMF. The residue was purified by column chromatography (pet. ether/EtOAc 7:1 to 2:1) to afford **5** and **6** with the yields of 66% and 84% respectively.

*5.2.2. General procedure for the preparation of targeted compounds **12**–**36***

Piperidine (0.08 mL) was added to methanol (25 mL) dissolved in substituted aldehydes **5**–**7** (0.18 mmol) and starting 2-indolinone derivatives **8**–**11** (0.18 mmol) which were prepared as described previously [6,21–25]. The mixture was refluxed for 5 h. For the targeted compounds **12**–**21** and **28**–**36**, the mixture was evaporated to remove the solvent and the residue was purified by column chromatography (CH_2Cl_2/MeOH 100:1–10:1). For the targeted compounds **22**–**27**, ethyl ether (15 mL) was added to the residue and the yellow precipitate was collected by filtration. The obtained precipitate was subjected to the hydrogen chloride saturated methanol to afford it as hydrochloride derivative. All the targeted compounds **12**–**36** show moderate to good yields under this reaction condition with the yields of 60–92%.

*5.2.2.1. (Z)-N-(2-(Dimethylamino)ethyl)-2,4-dimethyl-5-((2-oxo-1H-pyrrolo[3,2-b]pyridin-3(2H)-ylidene)methyl)-1H-pyrrole-3-carboxamide (**12**).* Yield 70%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.56 (1H, br s), 10.62 (1H, br s), 8.24 (1H, dd, J = 5.0, 1.5 Hz), 7.56 (1H, t, J = 5.5 Hz), 7.50 (1H, s), 7.28 (1H, dd, J = 8.0, 1.5 Hz), 7.22 (1H, dd, J = 7.5, 5.0 Hz), 3.32–3.30 (2H, m), 2.54 (3H, s), 2.41–2.38 (5H, m), 2.19 (6H, s); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.49, 164.96, 143.19, 140.04, 138.51, 136.12, 131.70, 126.53, 125.25, 122.24, 121.71, 116.51, 113.48, 58.72, 45.56, 37.34, 13.94, 10.98. ESI-MS m/z $[M+H]^+$ 354.

*5.2.2.2. (Z)-N-(2-(Dimethylamino)ethyl)-2,4-dimethyl-5-((2-oxo-1H-pyrrolo[3,2-c]pyridin-3(2H)-ylidene)methyl)-1H-pyrrole-3-carboxamide (**13**).* Yield 64%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.43 (1H, br s), 11.28 (1H, s), 8.91 (1H, s), 8.22 (1H, d, J = 5.5 Hz), 7.79 (1H, s), 7.54 (1H, t, J = 5.0 Hz), 6.90 (1H, d, J = 5.5 Hz), 3.30–3.28 (2H, m), 2.43 (3H, s), 2.40 (3H, s), 2.37 (2H, t, J = 6.5 Hz), 2.18 (6H, s); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.72, 164.93, 147.19, 144.46, 139.97, 137.29, 131.12, 126.42, 125.35, 122.40, 121.47, 112.01, 105.51, 58.73, 45.63, 37.32, 13.74, 10.99; ESI-MS m/z $[M+H]^+$ 354.

*5.2.2.3. (Z)-N-(2-(Diethylamino)ethyl)-2,4-dimethyl-5-((2-oxo-1H-pyrrolo[3,2-c]pyridin-3(2H)-ylidene)methyl)-1H-pyrrole-3-carboxamide (**14**).* Yield 62%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.50 (1H, br s), 11.36 (1H, s), 8.96 (1H, s), 8.28 (1H, d, J = 5.0 Hz), 7.85 (1H, s), 7.68–7.60 (1H, m), 6.96 (1H, d, J = 5.0 Hz), 3.41–3.39 (2H, m), 2.79–2.64 (6H, m), 2.50 (3H, s), 2.47 (3H, s), 1.07 (6H, t, J = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.51, 164.63, 146.95, 144.17, 139.76, 137.13, 130.77, 126.17, 125.06, 122.15, 121.14, 111.80, 105.23, 51.84, 46.74, 37.19, 13.54, 12.06, 10.81; ESI-MS m/z $[M+H]^+$ 382.

*5.2.2.4. (Z)-3-((4-(4-(2-Hydroxyethyl)piperazine-1-carbonyl)-3,5-dimethyl-1H-pyrrol-2-yl)methylene)-1H-pyrrolo[3,2-c]pyridin-2(3H)-one (**15**).* Yield 70%; mp 240–243 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.38 (1H, br s), 11.23 (1H, s), 8.89 (1H, s), 8.23 (1H, d, J = 5.0 Hz), 7.77 (1H, s), 6.90 (1H, d, J = 5.0 Hz), 4.39 (1H, t, J = 5.5 Hz), 3.49 (2H, q, J = 6.0 Hz), 3.37–3.70 (4H, m), 2.46–2.33 (4H, m), 2.40 (2H, t, J = 6.0 Hz), 2.26 (3H, s), 2.29 (3H, s); ESI-MS m/z $[M+H]^+$ 396.

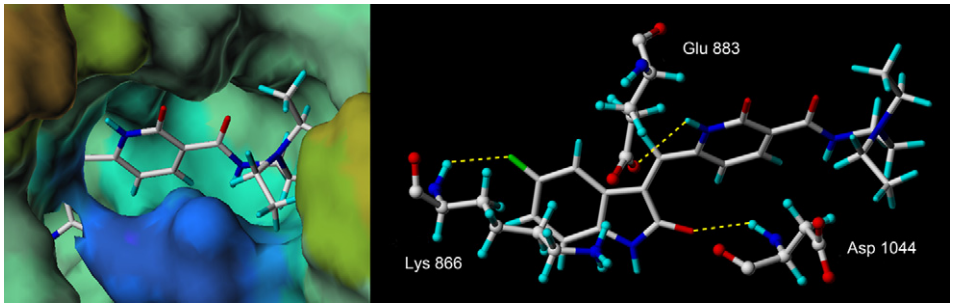


Fig. 5. Binding models of compound **23** bound to VEGFR-2 kinase (PDB entry 1Y6A) and potential interactions between compound **23** and Glu883, Lys866, Asp1044 in VEGFR-2 active site. The hydrogen bonds are labeled as yellow broken lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*5.2.2.5. (Z)-3-((3,5-Dimethyl-4-(4-methylpiperazine-1-carbonyl)-1H-pyrrol-2-yl)methylene-1H-pyrrolo[3,2-c]pyridin-2(3H)-one (**16**).* Yield 60%; mp 170–173 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.39 (1H, br s), 11.27 (1H, s), 8.90 (1H, s), 8.23 (1H, d, J = 5.0 Hz), 7.78 (1H, s), 6.90 (1H, d, J = 5.0 Hz), 3.75–3.44 (4H, m), 2.29 (3H, s), 2.26 (3H, s), 2.37–2.20 (4H, m), 2.17 (3H, s); ESI-MS m/z $[M+H]^+$ 366.

*5.2.2.6. (Z)-3-((4-(4-(2-Hydroxyethyl)piperazine-1-carbonyl)-3,5-dimethyl-1H-pyrrol-2-yl)methylene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one (**17**).* Yield 68%; mp > 250 °C ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.42 (1H, s), 11.47 (1H, s), 8.09 (1H, d, J = 7.5 Hz), 8.01 (1H, d, J = 5.0 Hz), 7.69 (1H, s), 7.00 (1H, dd, J = 7.5, 5.0 Hz), 4.44 (1H, t, J = 5.5 Hz), 3.48 (2H, q, J = 6.0 Hz), 3.70–3.41 (4H, m), 2.38 (2H, t, J = 6.0 Hz), 2.46–2.31 (4H, m), 2.28 (3H, s), 2.25 (3H, s); ESI-MS m/z $[M+H]^+$ 396.

*5.2.2.7. (Z)-2,4-Dimethyl-5-((2-oxo-1H-pyrrolo[2,3-b]pyridin-3(2H)-ylidene)methyl)-N-(2-(piperidin-1-yl)ethyl)-1H-pyrrole-3-carboxamide (**18**).* Yield 60%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.46 (1H, s), 11.46 (1H, s), 8.11 (1H, d, J = 7.5 Hz), 8.01 (1H, d, J = 5.0 Hz), 7.71 (1H, s), 7.51–7.42 (1H, m), 7.00 (1H, t, J = 6.0 Hz), 3.33–3.25 (2H, m), 2.44 (3H, s), 2.41 (3H, s), 2.40–2.24 (6H, m), 1.57–1.42 (4H, m), 1.42–1.30 (2H, m); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.75, 164.82, 153.10, 145.25, 137.22, 130.81, 126.24, 125.97, 125.63, 121.37, 120.37, 117.79, 113.09, 57.94, 54.38, 36.65, 26.08, 24.49, 13.75, 11.04. ESI-MS m/z $[M+H]^+$ 394.

*5.2.2.8. (Z)-2,4-Dimethyl-N-(2-morpholinoethyl)-5-((2-oxo-1H-pyrrolo[2,3-b]pyridin-3(2H)-ylidene)methyl)-1H-pyrrole-3-carboxamide (**19**).* Yield 64%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.47 (1H, s), 11.48 (1H, s), 8.12 (1H, d, J = 7.5 Hz), 8.02 (1H, d, J = 5.0 Hz), 7.72 (1H, s), 7.53 (1H, t, J = 5.5 Hz), 7.01 (1H, dd, J = 7.5, 5.0 Hz), 3.61–3.52 (4H, m), 3.33–3.29 (2H, m), 2.45 (3H, s), 2.47–2.43 (2H, m), 2.42 (3H, s), 2.43–2.36 (4H, m); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.75, 164.88, 153.10, 145.24, 137.16, 130.85, 126.24, 125.96, 125.62, 121.44, 120.36, 117.79, 113.07, 66.75, 57.72, 53.63, 36.27, 13.74, 11.01; ESI-MS m/z $[M+H]^+$ 396.

*5.2.2.9. (Z)-N-(2-(Dimethylamino)ethyl)-2,4-dimethyl-5-((2-oxo-1H-pyrrolo[2,3-b]pyridin-3(2H)-ylidene)methyl)-1H-pyrrole-3-carboxamide (**20**).* Yield 69%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.47 (1H, br s), 11.47 (1H, s), 8.10 (1H, dd, J = 7.5, 1.0 Hz), 8.01 (1H, dd, J = 5.0, 1.0 Hz), 7.70 (1H, s), 7.56 (1H, t, J = 5.5 Hz), 7.00 (1H, dd, J = 7.5, 5.0 Hz), 3.35–3.31 (2H, m), 2.48–2.46 (2H, m), 2.43 (3H, s), 2.39 (3H, s), 2.26 (6H, s); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.77, 165.81, 153.14, 145.37, 137.70, 131.07, 126.26, 126.16, 125.65, 120.30, 120.23, 117.84, 113.48, 56.70, 43.02, 34.74, 14.11, 11.27; ESI-MS m/z $[M+H]^+$ 354.

*5.2.2.10. (Z)-3-((3,5-Dimethyl-4-(4-methylpiperazine-1-carbonyl)-1H-pyrrol-2-yl)methylene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one (**21**).* Yield 67%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.42 (1H, s), 11.47 (1H, s), 8.10 (1H, dd, J = 7.5, 1.0 Hz), 8.01 (1H, dd, J = 5.0, 1.0 Hz), 7.69 (1H, s), 7.00 (1H, dd, J = 7.5, 5.0 Hz), 3.74–3.40 (4H, m), 2.34–2.20 (4H, m), 2.28 (3H, s), 2.25 (3H, s), 2.17 (3H, s); ESI-MS m/z $[M+H]^+$ 366.

*5.2.2.11. (Z)-N-(2-(Diethylamino)ethyl)-2-oxo-6-((2-oxo-1H-pyrrolo[3,2-b]pyridin-3(2H)-ylidene)methyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (**22**).* Yield 75%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 15.27 (1H, br s), 11.03 (1H, br s), 9.85 (1H, t, J = 5.5 Hz), 8.40 (1H, d, J = 7.5 Hz), 8.30 (1H, dd, J = 5.0, 1.5 Hz), 7.46–7.43 (3H, m), 7.21 (1H, d, J = 7.5 Hz), 3.42–3.38 (2H, m), 2.59–2.54 (6H, m), 0.99 (6H, t, J = 7.5 Hz); ESI-MS m/z $[M+H-HCl]^+$ 382.

*5.2.2.12. (Z)-N-(2-(Diethylamino)ethyl)-6-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2-oxo-1,2-dihydropyridine-3-carboxamide hydrochloride (**23**).* Yield 77%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.44 (1H, s), 11.49 (1H, s), 9.93–10.00 (1H, br s), 9.92 (1H, t, J = 6 Hz), 8.45 (1H, d, J = 7.5 Hz), 7.82 (1H, s), 7.74 (1H, dd, J = 8.5, 2.5 Hz), 7.20–7.16 (1H, m), 6.95–6.93 (2H, m), 3.71 (2H, q, J = 6.5 Hz), 3.27–3.23 (2H, m), 3.23–3.16 (4H, m), 1.23 (6H, t, J = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.29, 164.05, 161.68, 158.84 (d, J = 235.6 Hz), 144.22, 143.85, 138.44, 131.98, 129.50, 125.29 (d, J = 9.2 Hz), 122.72, 117.94 (d, J = 24.4 Hz), 114.50, 112.12 (d, J = 8.0 Hz), 109.17 (d, J = 25.8 Hz), 49.99, 47.36, 34.34, 8.96; ESI-MS m/z $[M+H-HCl]^+$ 399.

*5.2.2.13. (Z)-N-(2-(Dimethylamino)ethyl)-6-((5-fluoro-2-oxoindolin-3-ylidene)methyl)-2-oxo-1,2-dihydropyridine-3-carboxamide hydrochloride (**24**).* Yield 79%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.44 (1H, s), 11.46 (1H, s), 9.91 (1H, t, J = 6.0 Hz), 8.46 (1H, d, J = 7.5 Hz), 7.81 (1H, s), 7.74 (1H, dd, J = 8.5, 2.5 Hz), 7.21–7.17 (1H, m), 6.96–6.93 (2H, m), 3.68 (2H, q, J = 6.0 Hz), 3.22–3.17 (2H, m), 2.77 (6H, s); ESI-MS m/z $[M+H-HCl]^+$ 371.

*5.2.2.14. (Z)-6-((5-Fluoro-2-oxoindolin-3-ylidene)methyl)-N-(2-morpholinoethyl)-2-oxo-1,2-dihydropyridine-3-carboxamide hydrochloride (**25**).* Yield 80%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.36 (1H, s), 11.44 (1H, s), 9.86 (1H, t, J = 5.5 Hz), 8.43 (1H, d, J = 7.5 Hz), 7.78 (1H, s), 7.72 (1H, dd, J = 8.5, 2.5 Hz), 7.19–7.15 (1H, m), 6.92–6.89 (2H, m), 3.57 (4H, t, J = 4.5 Hz), 3.43 (2H, q, J = 6 Hz), 2.45 (2H, t, J = 6.0 Hz), 2.36–2.43 (4H, m); ESI-MS m/z $[M+H-HCl]^+$ 413.

*5.2.2.15. (Z)-6-((5-Fluoro-2-oxoindolin-3-ylidene)methyl)-2-oxo-N-(2-(piperidin-1-yl)ethyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (**26**).* Yield 78%; mp > 250 °C ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.38 (1H, s), 11.46 (1H, s), 9.87 (1H, t, J = 5.0 Hz), 8.45 (1H, d, J = 7.0 Hz), 7.80 (1H, s), 7.76 (1H, dd, J = 9.0, 2.5 Hz), 7.21–7.17 (1H, m), 6.95–6.92 (2H, m), 3.52–3.41 (2H, m), 2.46–2.34 (4H, m), 1.60–1.48 (4H, m), 1.48–1.35 (2H, m); ESI-MS m/z $[M+H-HCl]^+$ 411.

*5.2.2.16. (Z)-N-(2-(Dimethylamino)ethyl)-2-oxo-6-((2-oxo-1H-pyrrolo[2,3-b]pyridin-3(2H)-ylidene)methyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (**27**).* Yield 70%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 14.26 (1H, s), 12.02 (1H, br s), 9.91 (1H, t, J = 6.0 Hz), 8.47 (1H, d, J = 7.5 Hz), 8.23 (1H, dd, J = 5.0, 1.5 Hz), 8.14 (1H, dd, J = 7.5, 1.5 Hz), 7.83 (1H, s), 7.16 (1H, dd, J = 7.5, 5.0 Hz), 7.00 (1H, dd, J = 7.5, 1.5 Hz), 3.70 (2H, q, J = 6.0 Hz), 3.24 (2H, t, J = 5.0 Hz), 2.80 (6H, s); ESI-MS m/z $[M+H-HCl]^+$ 354.

*5.2.2.17. (Z/E)-3-((5-Fluoro-1H-indol-3-yl)methylene)-1H-pyrrolo[3,2-b]pyridin-2(3H)-one (**28**).* Yield 86%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.34 (1H, br s, *Z*), 10.70 (1H, s, *Z*), 9.59 (1H, s, *Z*), 8.33 (1H, s, *Z*), 8.14 (1H, dd, J = 4.5, 1.0 Hz, *Z*), 7.79 (1H, dd, J = 10, 2.5 Hz, *Z*), 7.57 (1H, dd, J = 9.0, 5.0 Hz, *Z*), 7.19–7.17 (1H, m, *Z*), 7.14–7.12 (2H, m, *Z*); 12.33 (0.4 × 1H, br s, *E*), 10.58 (0.4 × 1H, s, *E*), 10.09 (0.4 × 1H, s, *E*), 8.30 (0.4 × 1H, dd, J = 5.0, 1.5 Hz, *E*), 8.02 (0.4 × 1H, s, *E*), 7.77 (0.4 × 1H, dd, J = 10, 2.5 Hz, *E*), 7.57 (0.4 × 1H, dd, J = 9.0, 5.0 Hz, *E*), 7.23 (0.4 × 1H, dd, J = 7.5, 1.5 Hz, *E*), 7.19–7.17 (0.4 × 1H, m, *E*), 7.12–7.10 (0.4 × 1H, m, *E*); ^{13}C NMR (125 MHz, DMSO-d_6): *Z*: δ 168.90, 158.98 (d, J = 233.9 Hz), 144.11, 141.56, 138.18, 137.01, 133.45, 130.50, 129.35 (d, J = 10.0 Hz), 122.12, 118.07, 115.58, 114.30 (d, J = 9.9 Hz), 112.57 (d, J = 4.2 Hz), 111.42 (d, J = 25.9 Hz), 103.97 (d, J = 24.1 Hz); ESI-MS m/z $[M+H]^+$ 280.

*5.2.2.18. (Z/E)-3-((6-Fluoro-1H-indol-3-yl)methylene)-1H-pyrrolo[3,2-b]pyridin-2(3H)-one (**29**).* Yield 88%; mp > 250 °C; ^{1}H NMR

(500 MHz, DMSO-d_6): δ 12.19 (1H, br s, *E*), 10.59 (1H, s, *E*), 10.02 (1H, s, *E*), 8.31 (1H, dd, *J* = 5.0, 1.5 Hz, *E*), 8.03 (1H, s, *E*), 7.96 (1H, dd, *J* = 8.5, 5.0 Hz, *E*), 7.38 (1H, dd, *J* = 9.5, 2.0 Hz, *E*), 7.23 (1H, dd, *J* = 8.0, 1.5 Hz, *E*), 7.19 (1H, dd, *J* = 8.0, 2.5 Hz, *E*), 7.13–7.09 (1H, m, *E*); 12.19 (0.2 × 1H, br s, *Z*), 10.71 (0.2 × 1H, s, *Z*), 9.53 (0.2 × 1H, s, *Z*), 8.35 (0.2 × 1H, s, *Z*), 8.14 (0.2 × 1H, dd, *J* = 5.0, 1.5 Hz, *Z*), 7.99 (0.2 × 1H, dd, *J* = 8.5, 5.0 Hz, *Z*), 7.38 (0.2 × 1H, dd, *J* = 9.5, 2.0 Hz, *Z*), 7.19 (0.2 × 1H, dd, *J* = 8.0, 2.5 Hz, *Z*), 7.14–7.09 (0.4 × 2H, m, *Z*); ^{13}C NMR (125 MHz, DMSO-d_6): *E*: δ 169.04, 160.00 (d, *J* = 235.2 Hz), 143.97, 141.75, 137.18, 136.93, 136.84 (d, *J* = 12.8 Hz), 130.41, 125.10, 122.25, 119.68 (d, *J* = 10.1 Hz), 118.40, 115.76, 112.26, 110.30 (d, *J* = 24.1 Hz), 99.37 (d, *J* = 25.5 Hz); ESI-MS *m*/*z* $[M + H]^+$ 280.

*5.2.2.19. (E/Z)-3-((7-Ethyl-1H-indol-3-yl)methylene)-1H-pyrrolo[3,-2-b]pyridin-2(3H)-one (**30**).* Yield 86%; mp 242–244 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.29 (1H, br s, *E*), 10.57 (1H, s, *E*), 10.06 (1H, s, *E*), 8.30 (1H, dd, *J* = 5.0, 1.5 Hz, *E*), 8.06 (1H, s, *E*), 7.76 (1H, d, *J* = 7.5 Hz, *E*), 7.25–7.15 (3H, m, *E*), 7.11 (1H, d, *J* = 7.5 Hz, *E*), 2.92 (2H, q, *J* = 7.5 Hz, *E*), 1.30 (3H, t, *J* = 7.5 Hz, *E*) 12.29 (0.2 × 1H, br s, *Z*), 10.67 (0.2 × 1H, s, *Z*), 9.54 (0.2 × 1H, s, *Z*), 8.37 (0.2 × 1H, s, *Z*), 8.14 (0.2 × 1H, dd, *J* = 5.0, 1.5 Hz, *Z*), 7.79 (0.2 × 1H, d, *J* = 8.0 Hz, *Z*), 7.24–7.11 (0.2 × 4H, m, *Z*), 2.92 (0.2 × 2H, q, *J* = 7.5 Hz, *Z*), 1.30 (0.2 × 3H, t, *J* = 7.5 Hz, *Z*); ^{13}C NMR (125 MHz, DMSO-d_6): *E*: δ 169.15, 144.15, 141.65, 136.76, 136.48, 135.54, 130.95, 128.77, 128.47, 122.42, 122.26, 122.02, 117.59, 115.92, 115.64, 112.71, 23.99, 14.91; ESI-MS *m*/*z* $[M + H]^+$ 290.

*5.2.2.20. (Z)-3-((7-Ethyl-1H-indol-3-yl)methylene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one (**31**).* Yield 87%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.13 (1H, br s), 11.10 (1H, s), 9.43 (1H, d, *J* = 3.0 Hz), 8.22 (1H, s), 8.19 (1H, dd, *J* = 7.5, 1.0 Hz), 8.04–7.99 (2H, m), 7.21–7.16 (1H, m), 7.09 (1H, d, *J* = 7.0 Hz), 7.01 (1H, dd, *J* = 7.5, 5.0 Hz), 2.91 (2H, q, *J* = 7.5 Hz), 1.30 (3H, t, *J* = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.08, 153.80, 145.54, 135.06, 134.36, 131.10, 128.63, 128.41, 125.89, 122.01, 121.82, 120.54, 117.39, 117.12, 116.56, 112.26, 23.99, 14.95; ESI-MS *m*/*z* $[M + H]^+$ 290.

*5.2.2.21. (Z)-3-((5-Fluoro-1H-indol-3-yl)methylene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one (**32**).* Yield 85%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.20 (1H, s), 11.13 (1H, s), 9.47 (1H, s), 8.21 (1H, s), 8.12–8.00 (2H, m), 7.59–7.50 (1H, m), 7.15–7.05 (1H, m), 7.05–6.99 (1H, m); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.07, 158.85 (d, *J* = 232.4 Hz), 153.94, 145.70, 136.11, 132.98, 129.73, 129.61 (d, *J* = 10.5 Hz), 126.08, 120.50, 117.50, 117.39, 114.05 (d, *J* = 9.5 Hz), 112.11 (d, *J* = 3.9 Hz), 111.21 (d, *J* = 25.6 Hz), 104.43 (d, *J* = 24.1 Hz); ESI-MS *m*/*z* $[M + H]^+$ 280.

*5.2.2.22. (Z)-3-((6-Fluoro-1H-indol-3-yl)methylene)-1H-pyrrolo[2, 3-b]pyridin-2(3H)-one (**33**).* Yield 83%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.12 (1H, s), 11.12 (1H, s), 9.41 (1H, s), 8.22 (1H, s), 8.21–8.16 (2H, m), 8.03 (1H, dd, *J* = 5.0, 1.0 Hz), 7.33 (1H, dd, *J* = 9.5, 2.0 Hz), 7.09–7.13 (1H, m),7.01 (1H, dd, *J* = 7.0, 5.0 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.00, 159.98 (d, *J* = 230.4 Hz), 153.99, 145.80, 136.33 (d, *J* = 13.0 Hz), 135.07, 129.62, 126.12, 125.33, 120.41, 120.26 (d, *J* = 9.9 Hz), 117.96, 117.39, 111.82, 109.74 (d, *J* = 24.1 Hz), 99.08 (d, *J* = 25.5 Hz); ESI-MS *m*/*z* $[M + H]^+$ 280.

*5.2.2.23. (Z)-5-Fluoro-3-((5-fluoro-1H-indol-3-yl)methylene)indolin-2-one(**34**).* Yield 88%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.16 (1H, br s), 10.54 (1H, s), 9.49 (1H, s), 8.19 (1H, s), 8.11 (1H, dd, *J* = 10.0, 2.0 Hz), 7.87 (1H, dd, *J* = 9.5, 2.5 Hz), 7.34 (1H, dd, *J* = 9.0, 4.5 Hz), 7.11–7.07 (1H, m), 6.97–6.92 (1H, m), 6.80 (H, dd, *J* = 8.5, 4.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.66, 158.86 (d, *J* = 232.8 Hz), 158.46 (d, *J* = 232.4 Hz), 136.08, 135.82, 132.97, 129.73 (d, *J* = 10.1 Hz), 129.18, 127.74 (d, *J* = 9.1 Hz), 119.40 (d, *J* = 2.8 Hz), 113.93 (d, *J* = 9.8 Hz), 113.26 (d, *J* = 23.9 Hz), 112.05 (d, *J* = 4.4 Hz), 111.10 (d, *J* = 26.1 Hz), 109.99 (d, *J* = 8.4 Hz), 106.66 (d, *J* = 25.2 Hz), 104.50 (d, *J* = 24.1 Hz); ESI-MS *m*/*z* $[M + H]^+$ 297.

*5.2.2.24. (Z)-5-Fluoro-3-((5-fluoro-1H-indol-3-yl)methylene)indolin-2-one (**35**).* Yield 92%; mp > 250 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.09 (1H, br s, *Z*), 10.55 (1H, s, *Z*), 9.45 (1H, s, *Z*), 8.25 (1H, dd, *J* = 9.0, 5.5 Hz, *Z*), 8.21 (1H, s, *Z*), 7.86 (1H, dd, *J* = 9.5, 2.5 Hz, *Z*), 7.34 (1H, dd, *J* = 10.0, 2.5 Hz, *Z*), 7.14–7.10 (1H, m, *Z*), 6.97–6.93 (1H, m, *Z*), 6.81 (H, dd, *J* = 8.0, 4.5 Hz, *Z*); ^{13}C NMR (125 MHz, DMSO-d_6): *Z*: δ 168.61, 159.96 (d, *J* = 235. 0 Hz), 158.43 (d, *J* = 232.5 Hz), 136.31 (d, *J* = 12.5 Hz), 135.84, 135.04, 129.10, 127.61 (d, *J* = 9.2 Hz), 125.42, 120.30 (d, *J* = 10.0 Hz), 119.81 (d, *J* = 2.8 Hz), 113.37 (d, *J* = 23.9 Hz), 111.75, 110.01 (d, *J* = 8.2 Hz), 109.65 (d, *J* = 24.0 Hz), 106.64 (d, *J* = 25.2 Hz), 99.48 (d, *J* = 25.8 Hz); ESI-MS *m*/*z* $[M + H]^+$ 296.

*5.2.2.25. (Z)-5-Ethyl-3-((5-fluoro-1H-indol-3-yl)methylene)indolin-2-one (**36**).* Yield 86%; mp 246–248 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.09 (1H, br s), 10.51 (1H, s), 9.46 (1H, s), 8.21 (1H, s), 8.06 (1H, d, *J* = 8.0 Hz), 7.85 (1H, dd, *J* = 9.0, 2.5 Hz), 7.18 (1H, t, *J* = 7.5 Hz), 7.08 (1H, d, *J* = 7.0 Hz), 6.97–6.90 (1H, m), 6.80 (1H, dd, *J* = 8.0, 5.0 Hz), 2.91 (2H, q, *J* = 7.5 Hz), 1.29 (3H, t, *J* = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.65, 158.42 (d, *J* = 232.1 Hz), 135.70, 135.04, 134.32, 129.62, 128.76, 128.32, 127.76 (d, *J* = 9.1 Hz), 121.91, 121.69, 119.02 (d, *J* = 2.8 Hz), 116.69, 113.11 (d, *J* = 23.9 Hz), 112.17, 109.92 (d, *J* = 8.2 Hz), 106.48 (d, *J* = 25.1 Hz), 24.00, 14.96; ESI-MS *m*/*z* $[M + H]^+$ 307.

5.3. Cell culture

Various human cancer cell lines were cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum (Gibco Laboratories, Grand island, NY), 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere in 5% CO_2 at 37 °C. Cell culture media were renewed every three days, up to the confluence of the monolayer. Cell culture was washed upon formation of confluent cultures, using trypsin–EDTA to detach the cells from their culture flasks or dishes. Test compounds were stored at −70 °C and solubilized in 100% DMSO.

5.4. MTT assay for cell viability

Exponentially growing cells were seeded into 96-well microculture plates at a concentration of 1×10^4 cells per well. After 6 h incubation, cells were exposed to compounds at concentrations from 50 to 1.562 μM. After 48 h, 10 μL of MTT solution (5 mg/mL in phosphate buffered solution) was added to the culture medium and incubated at 37 °C for a further 4 h. After removing unconverted MTT, 200 μl of DMSO was added to each well and the plates shaken to dissolve the reduced MTT crystals (formazan); the optical density was measured on a microplate reader at a wavelength of 570 nm. The average 50% inhibitory concentration (IC_{50}) was evaluated by MTT tetrazolium dye assay. Each experiment was performed three times.

5.5. Flow cytometric analysis

HCT-116 Cells (1×10^5/mL) were seeded in a 6-well culture plate, after 24 h incubation, cells were treated with 0, 0.5, 2.0 and 8.0 μM of compound **23** for 48 h. Cells were collected and washed three times with D-Hank's buffer, re-suspended and fixed in 70% ice-cold ethanol for 2 h at 4 °C. Subsequently they were re-washed three times with D-Hank's buffer and treated with RNase A (100 μg/mL) and propidium iodide (PI) (20 μg/mL) for 30 min in the dark. Finally, cells were analyzed in a FACScan flow cytometer (BD

Biosciences, San Jose, CA, USA). The percentage of cells in G0/G1 phase, S phase, G 2/M and sub-G 1 phase was analyzed. To determine the cell apoptosis, fluorescent probes Annexin V/propidium iodide (PI, BioVision, CA, USA) were used following manufacturer's instructions.

5.6. Immunoblots analyses

HCT-116 cells (5×10^5/well) were pre-treated with indicated concentrations of compound for 30 min followed by 50 ng/mL VEGF for 2 h. The cells were harvested and re-suspended in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 μg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride). The lysates were centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. Equivalent amounts of proteins were analyzed by SDS-PAGE. The following appropriate primary antibodies to VEGFR and p-VEGFR from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA) were used. Proteins were visualized with peroxidase-coupled secondary antibody from Southern Biotech (Birmingham, UK), using ECL-plus kit from Amersham Biosciences (UK) for detection. Actin controls were used to normalize protein loading.

5.7. In vitro VEGFR-2 kinase assay

Into a black 96-well Costar plate was added 2 μL/well of compound in DMSO. An amount of 0.5 mmol pGAT-biotin and 3–4 ng hVEGFR-2 was added to each well in the presence of 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). The kinase reaction was initiated by addition of 10 μL of 10 μM ATP to reaction buffer after 10 min preincubation and the plate is incubated for additional 45 min at room temperature. The reaction was stopped by the addition of 100 mM EDTA and 0.36 μg/mL PY20 K in the presence of 50 μL KF buffer (50 mM Hepes, pH 7.5, 0.5 M KF, 1 mg/mL BSA). After 30 min, 100 μL of 10 nM SV-XL in KF buffer was added, and after an additional 2 h incubation at room temperature, the plate is read on the BMG Nova star.

5.8. Molecular modeling

The crystal structure of VEGFR-2 kinase in complex with a 2-anilino-5-aryl-oxazole inhibitor was downloaded from the Protein Data Bank (PDB entry 1Y6A) [30]. All calculations and manipulations were performed using SYBYL 6.9, running on SGI O2 workstation. Compound **23** was built using the SYBYL Sketcher model and fully minimized with the Powell method (Tripos force field and Gasteiger–Huckel charges) to an energy gradient of 0.001 kcal/(mol Å). For the *FlexX* docking calculation, *default parameters were used* and 30 binding modes were generated and ranked according to FlexX scoring function. The docking mode was chosen on the basis of binding affinity rank.

5.9. Statistical analysis

All data were expressed as mean ± S.D from at least three independent experiments. Differences between groups were examined for statistical significance using one-way ANOVA analysis with SPSS 10.0 for WINDOWS. In all cases, $P < 0.05$ was considered significant.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.10.009. These data include MOL files and InChiKeys of the most important compounds described in this article.

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