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Synthesis and Biological Evaluation of Novel Fluorinated Anticancer Agents Incorporating Indolin-2-One Moiety

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A series of novel fluorinated anticancer agents containing indolin-2-one moiety were designed, synthesized and evaluated for their anticancer activities *in vitro*. Among them, compounds 6, 7, 9, 12 and 13 showed potent activities against the tested human cancer cell lines. Notably, compound 6 showed significant activities against A549, Bel7402, HepG2, HCT116 and HeLa cancer cell lines, which were comparable to those of sunitinib. The further study on its mechanisms demonstrated that compound 6 can be used as a potential anti-cancer agent for inhibiting proliferation and inducing apoptosis of HepG2 cells, along with inhibiting angiogenesis of HUVECs.

ancer is a one of the most serious diseases and has gradually become a major public health problem all over the world. It is accounted for 8.2 million cancer-related deaths occurred in 2012 according to the WHO report. The incidence of cancer has been increasing in most regions of the world and new cancer cases will further increase to 19.3 million per year by 2025.[1] Although many effective chemotherapeutic agents have been approved to treat cancer, therapies for it are less satisfactory due to side effects. Therefore, searching for and developing novel effective agents for treating this disease are still urgently needed today. Various studies have been conducted to synthesize and find novel effective anticancer agents.[2-9]

Bromophenols possess various potent activities including protein tyrosine phosphatase 1B inhibitory, antioxidative, antimicrobial, proteintyrosine kinase (PTK) inhibitory, anticancer, antithrombotic, and antiinflammatory.[10] In our previous study, a series bromophenols isolated from marine algae with potent anticancer activities were reported.[11] The structural scaffold of indolin-2-one is proven as a core structure in anticancer active compounds. Those potent indolin-2-one analogs have aroused great attention for their anticancer activities and were developed as anticancer drugs, such as SU5416, SU5402, SU6668, SU14813, SU11248 (sunitinib), SU11274 and PHA665752 (Fig. 1).[12,13] Based on the anticancer pharmacophore moiety of natural bromophenol and indolin-2-one, a series of bromophenol derivatives containing indolin-2-one moiety were designed and synthesized. Seven compounds showed potent activity against the tested fives human cancer cell lines. Among of them, compound **WLJ18** (Fig. 1) was demonstrated that inactivated invasion and metastasis by inhibiting the migration of cancer cells.[14]

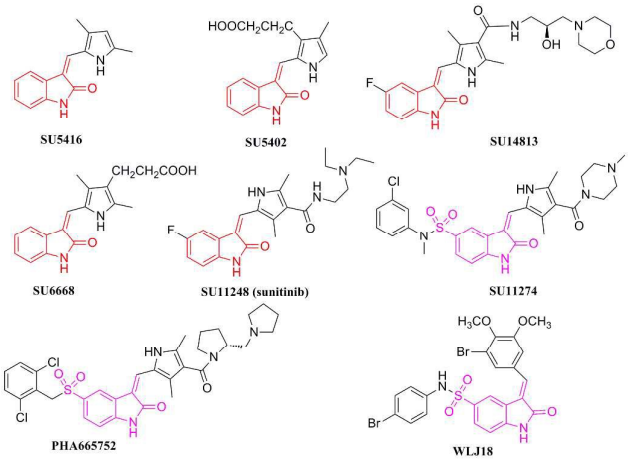

Fig. 1 Structures of indolinone derivatives as anticancer agents in preclinical or clinical trials and compound **WLJ18**.

Introduction of fluorine into drug has positive effects on several properties of molecules including: drug–target interactions and specificity, metabolic stability, acidity or basicity, membrane permeability and toxicity.[15-18] Based on structure–activity relationships (SARs) of bromophenol derivatives containing indolin-2-one moiety and F atom's important role in drug design, we designed and synthesized a

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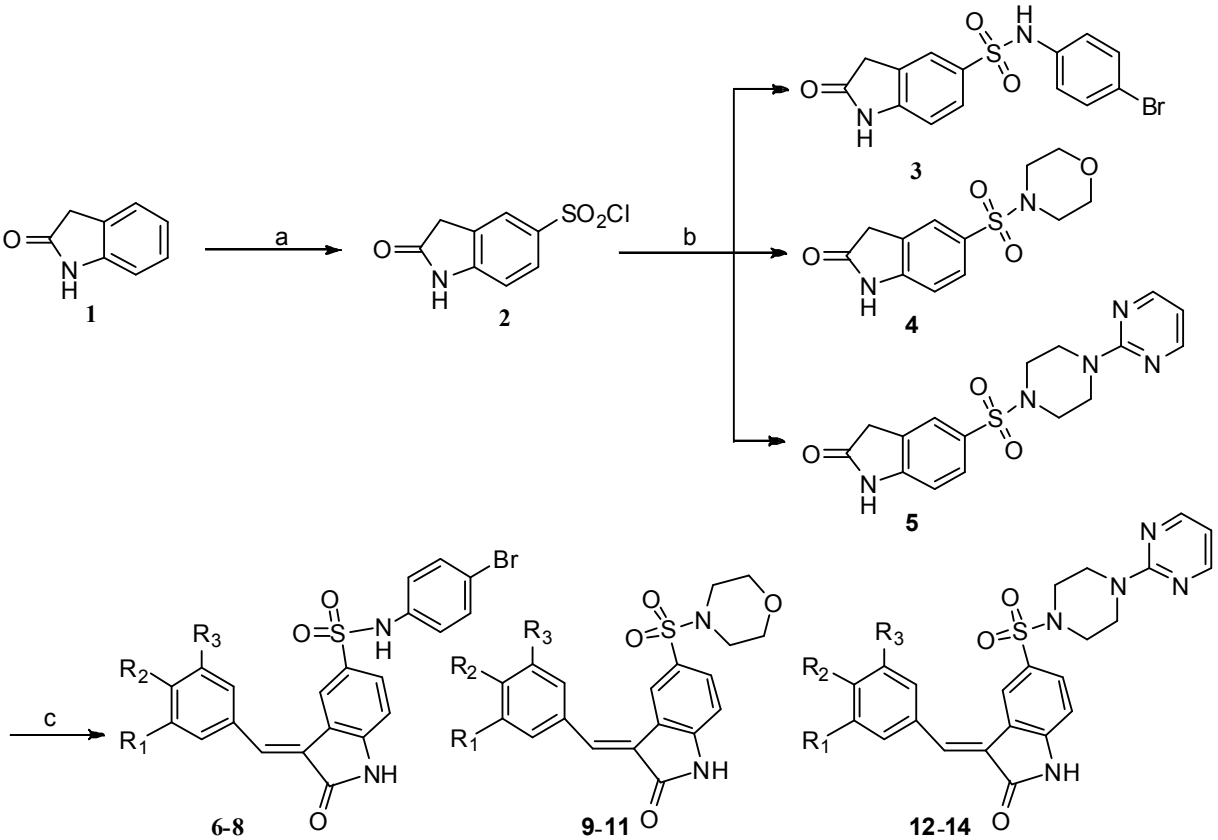
Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

series of fluorinated derivatives displaced Br using F atom in our continuing research for novel anticancer agents.



No.	R_1	R_2	R_3
6, **9**, **12**	CF_3	H	CF_3
7,**10**, **13**	F	F	H
8, **11**, **14**	F	OH	H

Scheme 1. Synthesis of compounds **6–14**. Reagents and conditions: (**a**) $ClSO_3H$, 65 °C, 1 h; (**b**) R_1R_2NH, THF, reflux; (**c**) EtOH, aldehyde, piperidine, reflux.

The general synthetic methods of compounds were shown in Scheme 1, which were performed as our prvious report.[14] Firstly, oxindole (**1**) was reacted with $ClSO_3H$ to yield compound **2**. And then, compound **2** and amines were heated for 3h in THF at 80 °C to afford 5-substituted-indolin-2-one (**3**-**5**). At last, the reaction between 5-substituted-indolin-2-one (**3**-**5**) and the aldehydes was performed under the condition of Knoevenagel condensation in ethanol with a catalytic amount of piperidine to give the desired derivatives **6-14** in good yields. All of the synthesized derivatives were purified and their structures were characterized by spectroscopic means (^1H, ^{13}C NMR, MS and HRMS). The configuration of the double bond in compounds **6-14** was assigned to E based on the spectra of ^1H NMR and referenced with Ref 19 and 20. Physicochemical properties (including cLogP-calculated logarithm of partition coefficient between n-octanol and water, TPSA-polar surface area, Ha-hydrogen bond acceptor, Hd-hydrogen bond donor) and toxicity profiles (including mutagenic effect, tumorigenic effect, irritating effect and reproductive effect) of these compounds were calculated and predicted using OSIRIS Property Explorer software at URL http://www.organic-chemistry.org/prog/peo/.[21] The calculation of physicochemical properties and prediction of toxicity risks were shown in table 1.

Table 1 Calculated physicochemical properties and predicted toxicity of compounds **6**-**14**

Compd	cLogP[a]	TPSA(Å^2)[b]	Ha [c]	Hd[d]	Toxicity Risks [e]
					M/T/I/R

WLJ18	3.88	102.1	7	2	L/L/L/H
6	4.99	83.65	5	2	L/L/L/H
7	3.49	83.65	5	2	L/L/L/H
8	3.05	103.8	6	3	L/L/L/H
9	3.01	84.09	6	1	L/L/L/H
10	1.51	84.09	6	1	L/L/L/H
11	1.06	104.3	7	2	L/L/L/H
12	3.42	103.8	8	1	L/L/L/H
13	1.92	103.8	8	1	L/L/L/H
14	1.47	124.1	9	2	L/L/L/H

[a]cLogP, calculated logarithm of partition coefficient between n-octanol and water; [b] TPSA, polar surface area; [c] Ha, hydrogen bond acceptor; [d]Hd, hydrogen bond donor; [e] Toxicity risks: M, mutagenic effect; T, tumorigenic effect; I, irritating effect; R, reproductive effect; L, low; M, medium; H, high.

All the newly synthesized compounds were investigated for their *in vitro* anti-cancer activity against five human cancer cell lines (human lung cancer cell line, A549; human hepatoma cell lines, Bel-7402 and HepG2; human cervical cancer cell line, HeLa; human colon cancer cell line, HCT116) by using MTT assay and sunitinib as a positive control. The preliminary results of inhibited ration of compounds **6**-**14** were summarized in Fig.2.

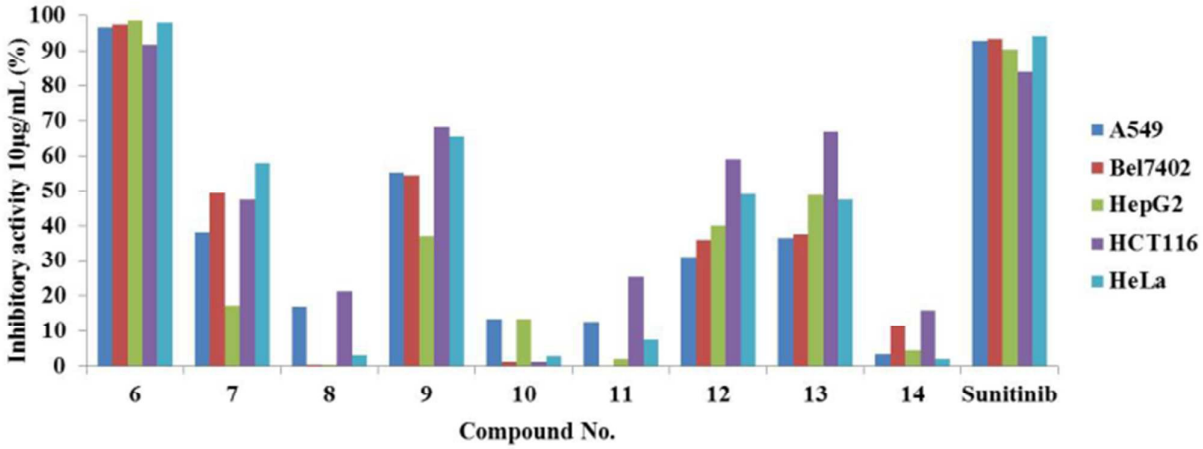


Fig.2 Newly synthesized compounds inhibit human cancer cell lines proliferation.

As shown in Fig.2, the synthesized compounds **6, 7, 9, 12**, and **13** exhibited potent anticancer activity against the tested human cancer cell lines at 10 μg/mL, respectively. Notably, compound **6** showed significant activity against A549, Bel7402, HepG2, HCT116 and HeLa cancer cell lines with the inhibition rate of 96.6%, 97.5%, 98.4%, 91.7% and 98.0% at 10 μg/mL, which were comparable to those of sunitinib. Compounds **7** and **9** showed better anticancer activity against A549, Bel7402, HCT116 and HeLa cancer cell lines than that of HepG2 cancer cell line at 10 μg/mL. Compounds **12** and **13** exhibited selective cytotoxicity to HCT116 cell line with 59.1% and 66.9% growth inhibitions at 10 μg/mL, and with under 50.0% inhibitions to A549, Bel7402, HepG2 and HeLa cancer cell lines, respectively.

Based on the preliminary bioassay results, the IC_{50} values were evaluated in order to investigate the potential anticancer activities. The IC_{50} values of the target compounds are presented in table 2. As shown in table 2, compound **6** exhibited excellent anticancer activities against A549, Bel7402, HepG2, HCT116 and HeLa cancer cell lines with IC_{50} values of 5.10 ± 0.64, 4.40 ± 0.78, 3.30 ± 0.14, 4.40 ± 1.09 and 4.30 ± 0.77 μg/mL, repectively, which better than that of our previous reported compound **WLJ18**. The results also testify that activities of compound **6** against A549, Bel7402, HepG2, HCT116 and HeLa cancer cell lines were comparable to those of sunitinib under the same conditions. Compound **7** showed potential inhibitory activity against HeLa cancer cell line with IC_{50} value 7.3 ± 0.87 μg/mL. Compound **9** displayed better anticaner activity against A549, Bel7402, HCT116 and HeLa cancer cell lines with IC_{50} values 7.2 ± 0.64 μg/mL, 8.86 ± 2.17 μg/mL, 5.23 ± 0.57 μg/mL, 5.3 ± 1.21 μg/mL than that of HepG2 cancer cell line (IC_{50} = 15.2 ± 1.76 μg/mL. Compounds **12** and **13** exhibited selective cytotoxicity to HCT116 cell line with IC_{50} values of 7.3 ± 1.26 μg/mL and 4.3 ± 0.82 μg/mL. The other compounds showed weak anticancer activities against the tested cancer cell lines.

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Table 2 IC_{50} values of compounds **6**-**14** against five human cancer cell lines.

Compd	IC_{50} (μg/mL)[a]				
	A549	Bel7402	HepG2	Hct116	Hela
WLJ18	6.6± 0.82	9.2± 0.84	13.2±2.42	9.1± 0.13	7.4± 0.22
6	5.10± 0.64	4.40± 0.78	3.30± 0.14	4.40± 1.09	4.30± 0.77
7	15.1± 2.13	15.8± 3.17	36.4± 0.74	11.3± 1.21	7.3± 0.87
8	NA[b]	NA	NA	NA	NA
9	7.2± 0.64	8.86± 2.17	15.2± 1.76	5.23± 0.57	5.3± 1.21
10	NA	NA	NA	NA	NA
11	NA	NA	NA	NA	NA
12	23.2 ± 3.11	14.5 ± 0.45	24.6 ± 1.78	7.3 ± 1.26	10.9 ± 1.53
13	15.0 ± 1.26	11.2± 2.03	12.6 ± 3.36	4.3 ± 0.82	11.3 ± 1.36
14	NA	27.1± 3.15	NA	NA	NA
Sunitinib[c]	11.7± 1.22	4.5± 0.56	4.9± 0.62	3.4± 1.26	2.6± 0.42

[a] IC_{50}: Concentration of the compound producing 50% cell growth inhibition after 48 h of drug exposure, as determined by the MTT assay. Each experiment was run at least three times, and the results are presented as average values ± standard deviation; [b] NA: Compound showing IC_{50} value > 50μg/mL; [c]Sunitinib as the positive control.

From above results, the preliminary SARs of these compounds can been drawn as the follows (Fig.3.): (I) the types of amino groups at 5-position of indolin-2-one could influence the activities; (II) the hydrophobic parameter was benefit for their anticancer activity; (III) the fluorinated moiety played a crucial role in maintaining anticancer activities of the conjugated derivatives.

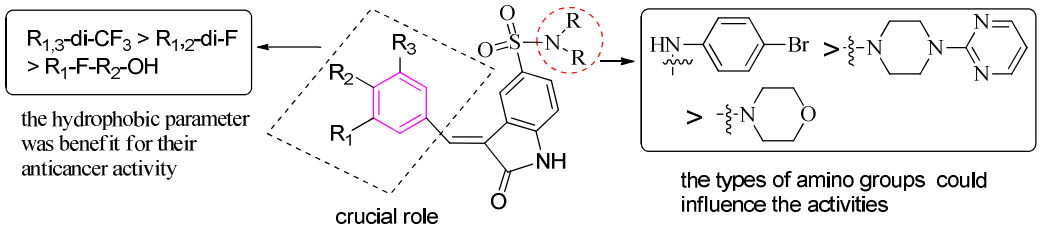

Fig. 3 Structure–Activity relationships of compounds **6**-**14**.

Along with proliferation inhibition of cancer cells, induction of apoptosis is an important strategy in cancer therapy. Thus, we checked the apoptotic effect of compound **6** by using a Hoechst33342 staining assay. After treated with compound **6** (0 μg/mL, 3μg/mL, 6μg/mL, 12μg/mL) for 12 h, the HepG2 cells were stained and photographed. As shown in Fig. 4, compound **6** induced apoptosis of HepG2 cells in a dose-dependent manner. Compound **6**, at the concentration of 12 μg/mL, was able to induce 25.75% cell apoptosis compared to the untreated one.

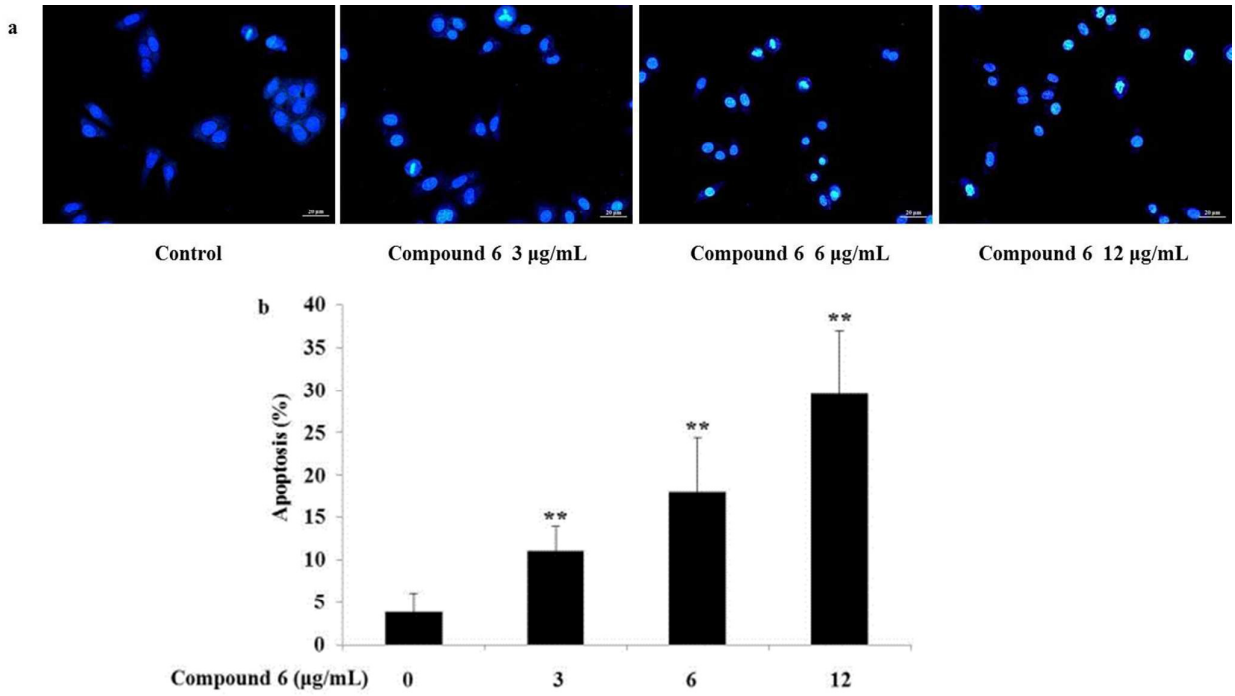

Fig. 4 Effect of compound **6** on apoptotic cell death by Hoechst33342 assay. (a)The cancer cells were treated with compound **6** for 12 h, and then stained with Hoechst 33342 solution. (b) The apoptotic cells were counted. The data shown in the graphs are the mean ± SD values of at least three individual experiments. ** p< 0.01 versus control.

To reveal the underlying mechanisms of apoptosis induced by compound **6**, HepG2 cells were treated with compound **6** at the indicated doses (0-12 μg/mL) for 12 h, and the apoptosis-related proteins were checked by immunoblotting assay. Compound **6** increased the pro-apoptotic protein expression of p53, Bax, cleaved caspase 9 and cleaved caspase 3. In contrast, compound **6** decreased the anti-apoptotic protein expression of Bcl-2 (Fig. 5). These results implied that compound **6** promoted apoptosis in HepG2 cells by regulating the apoptosis-related proteins.

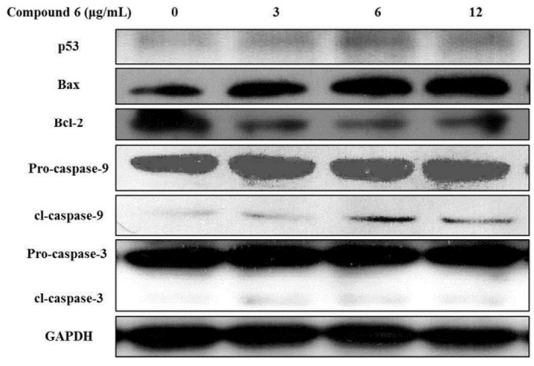

Fig. 5 Immunoblotting analysis of apoptosis related proteins of HepG2 cells treated with compound **6**.

Angiogenesis plays an important role in solid tumour growth. It has become a major target in exploiting anticancer drugs. For further understand the anticancer activity of compound **6**, we performed angiogenic assays in HUVECs. Before we started the angiogenesis assays, we checked the cytotoxicity of compound **6** on HUVECs. As shown in Fig. 6, compound **6** significantly induced cytotoxicity on HUVECs at 12 μg/mL concentration. Thus, we used **3** and **6** μg/mL of compound **6** in the next assays.

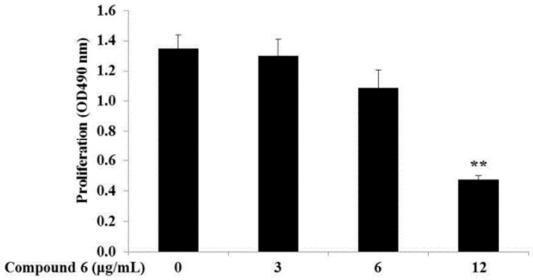

Fig. 6 Compound **6** (12 μg/mL) significantly inhibits HUVECs proliferation. ** p< 0.01 versus control.

Endothelial cells migration is one of the essential steps in the process of angiogenesis. We first investigated the migration of HUVECs in wound healing assay. HUVECs were wounded by sterile tips and incubated with 0, 3, 6 μg/mL of compound **6** for 24 h. The migration of HUVECs was significantly blocked by compound **6** at 3 and 6 μg/mL, which were shown in Fig.7.

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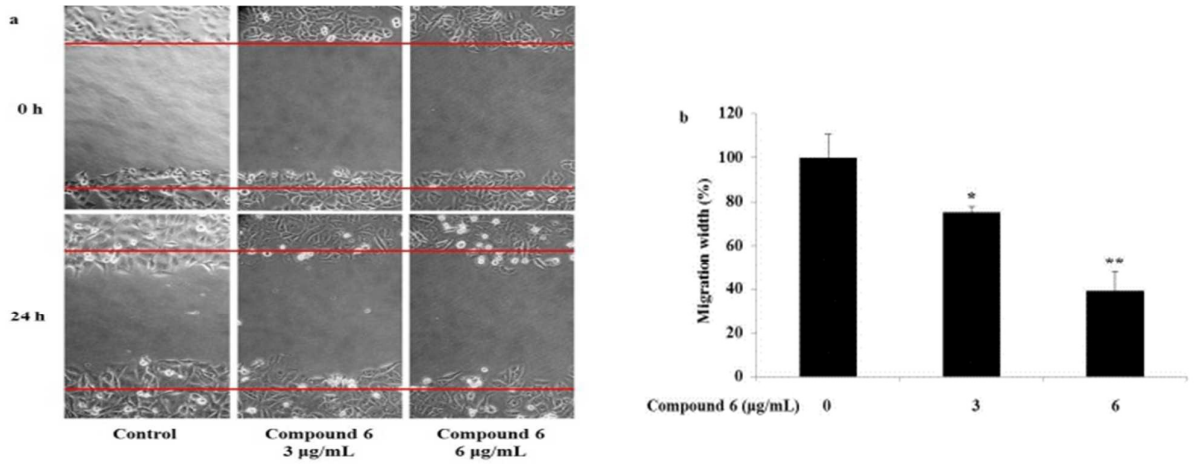

Fig. 7 HUVECs were plated at 6-well plate until 90% confluence. And then the HUVECs were scratched and treated with compound **6** as indicated concentrations for 24 h. (a) Images of the wounded monolayers of the HUVECs (200× magnification); (b) The migrated length of HUVECs. The data shown in the graphs are the mean ± SD values of at least three individual experiments. *p < 0.05 and **p < 0.01 compared to the control.

Tube formation is another major step of angiogenesis. Next, a tube formation assay was performed on growth factor enhanced Matrigel. As showed in Fig.8 (a and b), the total length of endothelial tubes formed on Matrigel was significantly reduced by compound **6** in a dose-dependent manner.

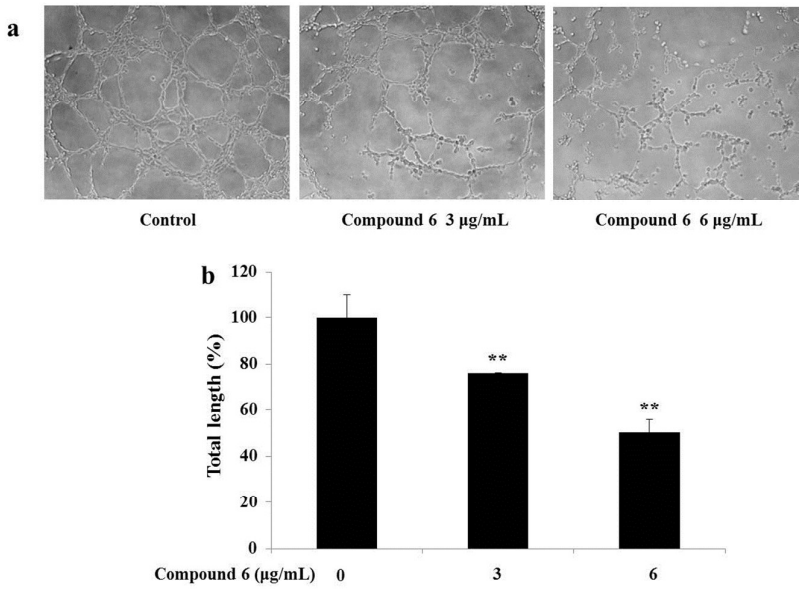

Fig. 8 HUVECs were seeded onto the growth factor enhanced Matrigel-coated 96-well plate with or without compound 6. (a) After 7 h incubation, the tube-like networks were photographed (200× magnification); (b) The length of the tube networks was quantified. The data shown in the graphs are the mean ± SD values of at least three individual experiments. ** p< 0.01 versus control.

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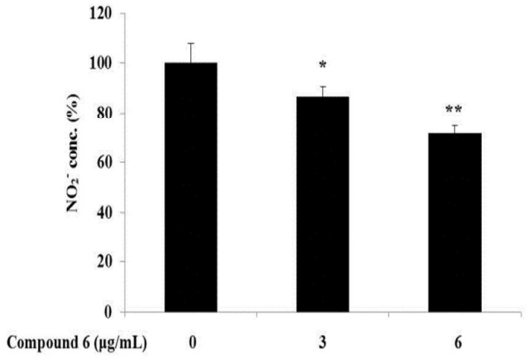

Fig. 9 HUVECs were incubated with 3–6μg/ml compound **6** for 24 h. Nitrite concentration was determined using the NO Kit. *p < 0.05 and **p < 0.01 compared to the control.

Nitric oxide (NO) is a signalling molecule that plays various roles pathologically and physiologically.[22] In endothelial cells, NO plays a key role in tumour angiogenesis. Finally, we checked the NO production in HUVECs after treated with compound **6**. The concentration of NO was significantly reduced after treating HUVECs for 24 h (Fig.9). Above all, compound **6** was considered to have an antiangiogenic activity in endothelial cells.

Conclusions

In summary, we synthesized a series of novel fluorinated anticancer agents containing indolin-2-one moiety and evaluated for their anticancer activities against A549, Bel7402, HepG2, HeLa and HCT116 cancer cell lines by using MTT assay *in vitro*. Among them, compounds **6**, **7**, **9**, **12** and **13** showed potent activity against the tested five human cancer cell lines. Notably, compound **6** showed significant activity against A549, Bel7402, HepG2, HCT116 and HeLa cancer cell lines with IC_{50} values of 5.10 ± 0.64, 4.40 ± 0.78, 3.30 ± 0.14, 4.40 ± 1.09 and 4.30 ± 0.77 μg/mL, which were comparable to those of sunitinib. The further study on its mechanism demonstrated that compound **6** can be used as a potential anticancer agent for inhibiting proliferation and inducing apoptosis of cancer cells, along with inhibiting angiogenesis of HUVECs. The further studies on structural modifications and related mechanism of action of these compounds are currently in progress, which will be reported in future.

Acknowledgements

The work was supported by the National Natural Science Foundation of China (No. 41276167, 41206066, 41506169 and 41306157), China Postdoctoral Science Foundation (No. 2015T80755 and 2014M551972), the National science and technology support project (2013BAB01B02-2), Science and Technology Development Program of Qingdao Shinan District (NO. 2013-12-008-SW), Basic Research Projects of Qingdao Science and Technology Plan (NO. 14-2-4-35-jch).

Author Contributions

Shuai-Yu Wang, Li-Jun Wang, and Da-Yong Shi contributed to the study concept and design, and the manuscript preparation. Li-Jun Wang and Shuai-Yu Wang performed the experimental studies and analyzed the data. Bo Jiang, Ning Wu, Xiang-Qian Li, Jiao Luo, Bao-Cheng Wang, Ren-Shuai Zhang and Qi Xu contributed in critical reading and discussion on the manuscript. All the authors approved the final version.

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23 Compound **6**: Yellow solid; ^{1}H NMR (Acetone-d_6, 500 MHz, ppm): δ10.17(s, 1H), 9.13(s, 1H), 8.38(s, 1H), 8.18(s, 1H), 8.12(s, 1H), 7.74(d, 1H, *J*= 8.0Hz),7.42(d, 2H, *J*= 8.5Hz), 7.20(d, 2H, *J*= 8.5Hz), 7.07(d, 1H, *J*= 8.0Hz); ^{13}C NMR (Acetone-d_6, 125 MHz, ppm): δ 166.7, 145.0, 137.7, 136.0, 135.0, 132.9, 132.1(2C), 131.9(2C), 131.1(2C), 129.4, 128.7, 124.8, 123.4(2C), 122.6(2C), 122.3, 119.6, 116.7, 110.0; ESIMS: *m/z* 588 $[M-H]^-$ HRESIMS: calc for $C_{23}H_{12}F_6BrN_2O_3S$ $[M-H]^-$ 588.9648, found 588.9662.

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