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Exploration of acridine scaffold as a potentially interesting scaffold for discovering novel multi-target VEGFR-2 and Src kinase inhibitors

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ABSTRACT

VEGFR-2 and Src kinases both play important roles in cancers. In certain cancers, Src works synergistically with VEGFR-2 to promote its activation. Development of multi-target drugs against VEGFR-2 and Src is of therapeutic advantage against these cancers. By using molecular docking and SVM virtual screening methods and based on subsequent synthesis and bioassay studies, we identified 9-aminoacridine derivatives with an acridine scaffold as potentially interesting novel dual VEGFR-2 and Src inhibitors. The acridine scaffold has been historically used for deriving topoisomerase inhibitors, but has not been found in existing VEGFR-2 inhibitors and Src inhibitors. A series of 21 acridine derivatives were synthesized and evaluated for their antiproliferative activities against K562, HepG-2, and MCF-7 cells. Some of these compounds showed better activities against K562 cells in vitro than imatinib. The structure–activity relationships (SAR) of these compounds were analyzed. One of the compounds (**7r**) showed low μM activity against K562 and HepG-2 cancer cell-lines, and inhibited VEGFR-2 and Src at inhibition rates of 44% and 8% at 50 μM, respectively, without inhibition of topoisomerase. Moreover, 10 μM compound **7r** could reduce the levels of activated ERK1/2 in a time dependant manner, a downstream effector of both VEGFR-2 and Src. Our study suggested that acridine scaffold is a potentially interesting scaffold for developing novel multi-target kinase inhibitors such as VEGFR-2 and Src dual inhibitors.

1. Introduction

Receptor and non-receptor tyrosine kinases (TKs) play critical roles in the regulation of cell growth, differentiation, development, etc.[1,2] The overexpression or dysregulation of TKs lead to multiple types of clinical disorders and diseases, including cancers.[1–4] Compounds against selected TKs have been developed and applied as potent anticancer agents.[1–9] In particular, vascular endothelial growth factor (VEGFR) is a member of the receptor TKs. VEGFR-mediated signaling plays a critical role in regulation of angiogenesis and survival.[10] VEGFR-2, one of VEGFR, promotes the main mitogenic, angiogenic, and takes part in developmental angiogenesis and hematopoiesis.[10,11] VEGFR-2 is frequently expressed or activated in various cancers, and VEGFR-2 inhibitors have been successfully developed for inhibiting tumor cell growth and clinically used for anticancer therapeutics.[12–14]

Apart from the roles of VEGFR in tumour cells, Src is also known to be involved in oncology.[15,16] Src kinase, which is present in the cytoplasm, is a member of non-receptor TKs.[17] Src plays an important role in modulating multiple pathways of cell proliferation, differentiation and migration. Because of its important roles, Src has been explored as an important target for the treatment of cancer.[15–19] Moreover, it has been reported that VEGF-stimulated recruitment and activation of Src enable the activation of beta3 integrin and its interaction with VEGFR-2 to promote the activation of VEGFR-2.[20,21] Therefore, Src works with VEGFR-2 synergistically to promote some cancers, and the development of multi-target drugs simultaneously against these two kinases likely offer a better therapeutic advantage.

Our work was intended to discover novel scaffolds by using molecular docking and SVM based virtual screening methods.[4] By using the two virtual screening methods to screen PubChem and in-house compound libraries, two compounds with an acridine scaffold was identified as virtual VEGFR-2 and Src dual inhibitors (Fig. 1). Acridine and its derivatives have been reported to display potent antitumor activity in vitro/vivo.[22–25] Primarily due to their inhibition of topoisomerases,[23] but no structure with this scaffold has been found in the known inhibitors of VEGFR-2 and Src before. This stimulated our interest to

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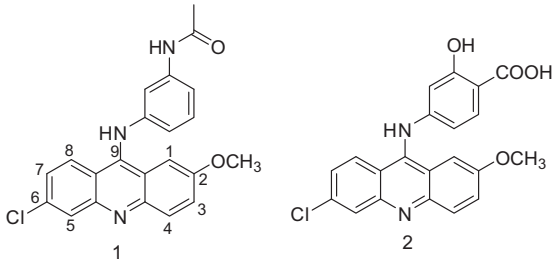


Figure 1. Structures of two acridine compounds as virtual VEGFR-2 and Src dual inhibitors.

investigate the possibility of exploring this scaffold for developing novel VEGFR-2 and Src dual inhibitors without inhibitory activity against topoisomerase, that is, to derive multi-target kinase inhibitors from a historical topoisomerase inhibitor scaffold. For this purpose, we designed and synthesized a series of acridine derivatives to investigate if they can be potentially developed as VEGFR-2 and Src dual inhibitors without inhibition of topoisomerase, and to analyze their structure–activity relationships.

2. Results and discussion

2.1. High-throughput virtual screening

Virtual screening against Pubchem and in-house libraries was conducted using the similar methods and computational procedures as those described in recent papers.[4,26] First, SVM models of VEGFR-2 inhibitors and Src inhibitors developed in earlier works[4] were used to initially screen the 13 million compounds in Pubchem database and 220 compounds in-house chemical libraries, then the initially selected SVM virtual hits were evaluated by Lipinsky's rule of five, those passed Lipinsky's rule of five were subject to further and more refined screening by using molecular docking software Discovery Studio.2.5/Libdock protocol. The in-house chemical libraries are composed of compounds of various scaffolds synthesized from our published[24,25,27–31] and yet-to-be published works. In particular, the acridine scaffold was synthesized as part of our efforts to expand our in-house libraries to cover more anticancer scaffolds that can be potentially explored for developing multi-target anticancer compounds.

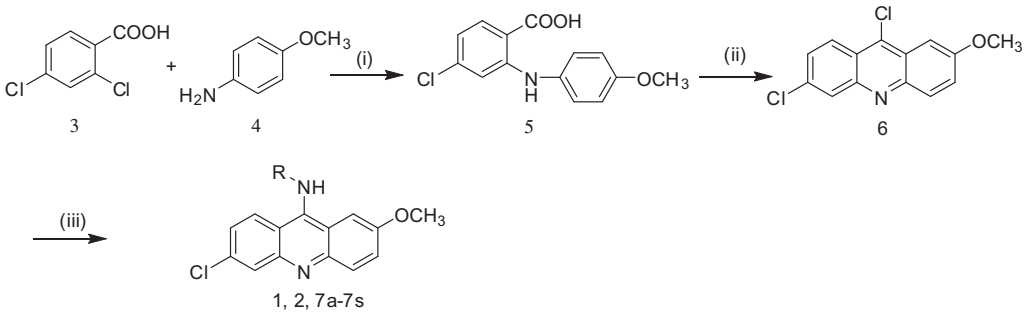
2.2. Chemistry

The synthetic pathway was illustrated in Scheme 1. The reaction of benzoic acid derivatives **3** with the anilines **4** in the presence of Cu afforded the corresponding anthranilic acid **5** (Ullmann conditions). Cyclization of compound **5** using $POCl_3$ gave the 9-chloroacridine derivatives **6**. The synthesis of compounds **1, 2, 7a–7n** was achieved in moderate yields by reacting 9-chloroacridine derivatives **6** with aniline derivatives in the presence of a few drops of concentrated hydrogen chloride; however, attempts to prepare compounds **7o–7q** containing pyridine moiety by this synthetic method failed due to their strong electron-withdrawing characteristics in acid conditions. The pyridine substituted acridine derivatives were obtained in high yields by reaction of **6** with the appropriate aminopyridine derivatives in the presence of NaH and catalytic amounts of potassium iodide in *N,N*-dimethylformamide, and compounds **7r–7s** where the alkylamine group was located at the C-9 position of the acridine ring were successfully synthesized by using this method.

2.3. In vitro cell growth inhibition assay

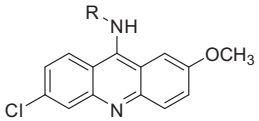
K562 leukemia cells express high levels of VEGFR-2[32] and Src[33] kinase, therefore, for the SAR study, the acridine derivatives synthesized were initially evaluated for antiproliferative activity against K562 leukemia cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, which is widely accepted for the screening of anticancer candidates, was used to determine the antiproliferation activity of the synthesized compounds. Imatinib was used as a reference compound. As can be seen in Table 1, multiple compounds have low μM IC_{50} values, some of which appear to show better activity than imatinib. It is noted that the two virtual VEGFR-2 and Src dual inhibitors identified by the virtual screening methods, compounds **1** and **2**, showed moderate inhibition activity. The observed cytotoxic activities show high sensitive to the substituted moiety of the C-9 position of the acridine ring.

When various aniline derivatives were attached to the framework of the acridine, the substitution pattern on the anilino ring was found to significantly influence the cytotoxicity. Compound **7a** with methoxy group on C4 position is the most potent compound among the 9-anilinoacridine derivatives, which is about 2.5 times more active than imatinib. In order to study the position of the methoxy group on the 9-anilinoacridine derivatives, we also prepared methoxy group positioned at the *meta*-(**7b**) or *ortho*-(**7c**) position. Although the results indicated that the cytotoxicity of these two compounds were less than that of **7a**, both of them showed high cytotoxic activity, comparable or superior to that of imatinib. By changing the methoxy to ethoxy group, compounds (**7d**, **7e**) showed decreasing inhibitory effect. The cytotoxic activities of **7e** and **7d** were about 1.5 less potent than that of **7a** and **7c**. Compound **7f** with three methoxy groups on the anilino ring displayed poor inhibitory effect on the tumour cell growth. These results suggest that increasing lipophilicity might decrease the cytotoxicity of this class. In addition, we also synthesized 9-anilinoacridines (**7g–7i**), which bear a hydroxyl or amino group. These compounds had a dissociable proton, which

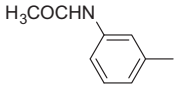
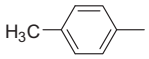
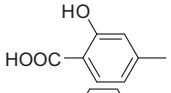
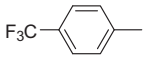
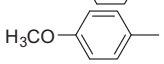
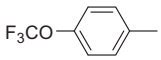
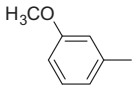
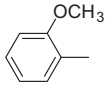
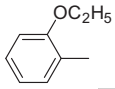
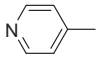
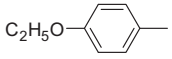
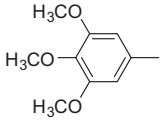
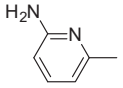
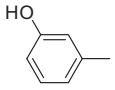
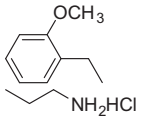
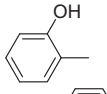
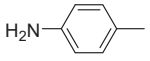


Scheme 1. Synthesis of the desired compounds (**1**, **2** and **7a–7s**). Reagents and conditions: (i) K_2CO_3, Cu, DMF, 130 °C; (ii) $POCl_3$, 140 °C; (iii) various anilines/amines, for **1**, **2** and **7a–7n** $CHCl_3$, EtOH, concd HCl, for **7o–7s** DMF, KI, NaH.

Table 1
Inhibitory activity of compounds against K562 cells



1,2,7a-7x

Compound	R	IC_{50} (μM)	Compound	R	IC_{50} (μM)
1		21.3	**7j**		5.5
2		18.7	**7k**		10.8
7a		2.2	**7l**		9.7
7b		2.7	**7m**		13.5
7c		5.4	**7n**		23.8
7d		9.0	**7o**		7.3
7e		3.2	**7p**		11.7
7f		31.0	**7q**		12.0
7g		5.5	**7r**		5.0
7h		4.9	**7s**		7.0
7i		7.7	Imatinib		5.4

might form a hydrogen bond with the targets. However, the results showed that these three compounds were all less cytotoxic than **7a**. To determine what effect the electron-negativity of the substitutes on the aniline ring has on cytotoxicity, compounds (**7j**–**7n**) were synthesized and tested their antiproliferative activity. Compound **7j** with methyl group in the aniline ring, the weaker electron-donating group, produced high activity (IC_{50}, 5.5 μM). Fluorine has a unique effect on the reactivity and compounds modified by fluorine may improve their antitumor activity. However, compound **7m** with fluoro group positioned at the *ortho*-position in the aniline ring was about 2.5-fold less potent than the corresponding compound **7c** with methoxy group. Compound **7n** with chloro group was about 1.6-fold less potent than **7m**. Compounds **7k** (fluoromethy) and **7l** (fluoromethoxy) exhibited similar IC_{50} values and they were about two-fold less potent than **7j**. Compound **2** with hydroxyl, the electron-donating group and carboxyl, the electron-withdrawing group was less potent than the corresponding compound **7g** with hydroxyl group. The results indicated that electron-withdrawing group introduced to the aniline ring might result in a decrease activity.

vIn addition, the pyridinyl amino group was also introduced to the C-9 position of the acridine ring to further study the SAR of the acridine derivatives. Compound **7o** with no substitute in the pyridine ring exhibited good cytotoxicity (IC_{50}, 7.3 μM), the toxicity of which toward K562 cells was a little lower than that of imatinib. Compounds **7p** and **7q** with amino group located at C-2 or C-3 position in the pyridinyl amino ring displayed similar antiproliferative activity, which appeared that the position of the amino might affect little on the cytotoxicity. The toxicity of them was about 1.5-fold less potent than **7o**. In another study, we replaced the anilino group with an alkyl amino moiety positioned at the C-9 position of the acridine ring (**7r** and **7s**). The in vitro study demonstrated that both of them showed good cytotoxicity comparable to that of imatinib.

The good in vitro activity of the compounds synthesized against K562 cell lines motivated us to investigate the antiproliferative potential effect of compounds against two solid tumour cells, HepG-2 and MCF-7 cells. The results were illustrated in Table 2, which showed that some of these new synthesized compounds also show low μM activities against the growth of these two solid tumour cell lines. Overall, the human leukemic K562 cells appear to be more sensitive to the new compounds than the human solid tumor cells (Table 1 and Table 2).

2.4. In vitro kinase and topoisomerase inhibition assay

In order to evaluate whether acridines synthesized can truly inhibit VEGFR-2 and Src, we conducted in vitro kinase inhibition assay studies on three of our synthesized compounds representative of the compound series we synthesized. Compound **2** with an anilino group is one of the two virtual hits from virtual screening methods. Compound **7r** with an alkane amino moiety represents compounds with best overall activities against K562 and HepG-2 cancer cell-lines. Compound **7q** containing pyridinyl amino group represents compounds with moderate activities against the cancer cell-lines. The kinase assay results are shown in Table 3. The pan-kinase inhibitor staurosporine was used as a positive reference compound. All the compounds were tested at 50 μM. It was found that among the three acridine derivatives tested, compound **7r** exhibited moderate activity with VEGFR-2 and Src inhibition rates of 44% and 8% at 50 μM, respectively, while the other two compounds showed weaker inhibition rates of 2.8–6.1% at 50 μM. Compounds **2** and **7r** with the lowest or best kinase activities were further evaluated to determine if they are topoisomerase inhibitors. Because the structure of our compounds are similar to AMSA, a topoisomerase II inhibitor clinical used for anticancer therapeutics, we conducted in vitro human topoisomerase II decatenation assay for measuring the possibly inhibitory activity of compound **2** and **7r** against topoisomerase II, which showed no activities (Supplementary data).

Moreover, the effect of 10 μM of compound **7r** on the phosphorylation of ERK1/2, a downstream effector of both VEGFR-2[34,35] and Src,[36] was performed on the K562 cells for 1, 2, and 4 h. As shown in Figure 2, **7r** reduced the levels of activated ERK1/2 in a time dependant manner consistent with those of VEGFR-2 inhibitors[34,35] and Src inhibitors,[36] eventhough, 7r is of substantially lower potency against each individual target VEGFR-2 and Src than the inhibitors reported in relevant studies. Taken together, the good activities of compound **7r** against K562 and HepG-2 cell-lines are likely due in main part to its inhibition of VEGFR-2 and Src, and our work suggests that it is possible to derive novel VEGFR-2 and Src dual kinase inhibitors from the acridine scaffold without inhibitory activity against topoisomerase.

Table 2
Antiproliferative activity of compounds against HepG-2 and MCF-7 cells

Compound	IC_{50} (μM)		Compound	IC_{50} (μM)	
	HepG-2	MCF-7		HepG-2	MCF-7
1	36.8	>50.0	**7j**	8.8	19.9
2	>50.0	>50.0	**7k**	>50.0	>50.0
7a	6.3	>50.0	**7l**	10.2	18.9
7b	14.9	>50.0	**7m**	46.1	>50.0
7c	>50.0	>50.0	**7n**	30.2	>50.0
7d	27.9	>50.0	**7o**	4.1	>50.0
7e	7.4	41.6	**7p**	10.6	23.2
7f	16.8	>50.0	**7q**	32.7	17.6
7g	9.6	13.2	**7r**	4.2	23.8
7h	11.4	>50.0	**7s**	6.0	9.3
7i	11.0	23.2	Colchicin	1.9	1.9

Table 3
Percent inhibition effect of compounds selected at 50 μM on the activity of two kinases

Compound	Src	VEGFR-2
2	2.80%	5.40%
7q	6.12%	3.77%
7r	8.02%	44.03%
Staurosporine	99.36%	99.58%

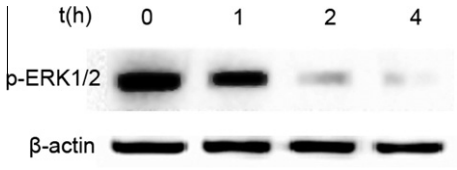


Figure 2. K562 cells were treated with 10 μM compound **7r** for the indicated times, lysed, and the levels of phosphorylated ERK1/2 proteins determined by Western blot analysis using specific antibodies.

2.5. Possible reasons for the difference in cell-line and in vitro kinase inhibition activities

The three tested acridines showed moderate to weak activities against the two kinases, indicative of the potential of acridine scaffold for developing novel VEGFR-2 and Src dual inhibitors. However, the best kinase activities of these acridines, particularly compound **7r**, appear to be 10 or more fold weaker than their low μM cytotoxic activities against K562 cell lines, and to a less extent the activities against HepG-2 cell lines. This discrepancy in potency may arise from one or more of the following possibilities. The first possibility is the synergistic effects of multi-target agents of weak or moderate activities. There are experimental evidences that multi-target agents with weak or moderate inhibition rates against their targets are capable of producing ~10-fold better cell-line activities. For instance, the multi-target VEGFR2 and Raf inhibitor CHIR-265/RAF-265 inhibits VEGFR2 and Raf at IC_{50} values of 1.3 μM and 1.2 μM, respectively,[37] while its IC_{50} values against SK-MEL-28, Malme-3 M and A375 M cell-lines are in the range of 140–300 nM.[38] The second possibility is that factors such as alternation of lipophilicity hydrophilicity balance by the substituents may affect the cytotoxic activity of these compounds.[39] The third possibility is that the cell-line activity of these compounds may be partly attributed to their additional activities against some other anticancer targets. Two indications suggest that, even if these compounds are active against additional targets, inhibition of VEGFR-2 and Src is likely an important factor for their low μM activities against K562 and HepG-2 cell lines. The first indication is that the cell-line structure–activity relationships of these compounds are comparable to that of combined kinase activities, with the compound of higher combined kinase activities having higher cell-line activity. The second indication is that these compounds showed no activity against the most-likely third target, topoisomerase.

2.6. Molecular docking study

To better understand whether the acridine compounds can interact with VEGFR-2 and Src, a molecular docking study was performed using the Discovery Studio 2.5/Libdock protocol. The crystal structure of VEGFR-2 with 2-fluoro-5-trifluoromethyl phenyl-urea was used as the template (PDB code 1YWN).[40] The representative binding result of compound **7r** was shown in Figure 3. Hydrogen bonding was observed between methoxy group and Cys917 with a distance of 2.284 Å. In addition, cation–π interaction was formed between the phenyl group of the acridine with an ammonium cation of Lys866. π–sigma interaction was also occurred between the phenyl group of **7r** with Val914 and Leu838 The LibDockScore is 89.174. The complexes obtained were used for molecular dynamics studies, and the result showed that compound **7r** could interact with the VEGFR-2 and the estimated free energy of binding was summarized in Table 4.

Compound **7r** was also docked onto the Src-binding domain. The X-ray structure of Src (PDB code 2H8H)[41] was selected as docking reference. Figure 3 depicted the interaction of the chemical structure **7r** and the Src kinase catalytic domain. The results

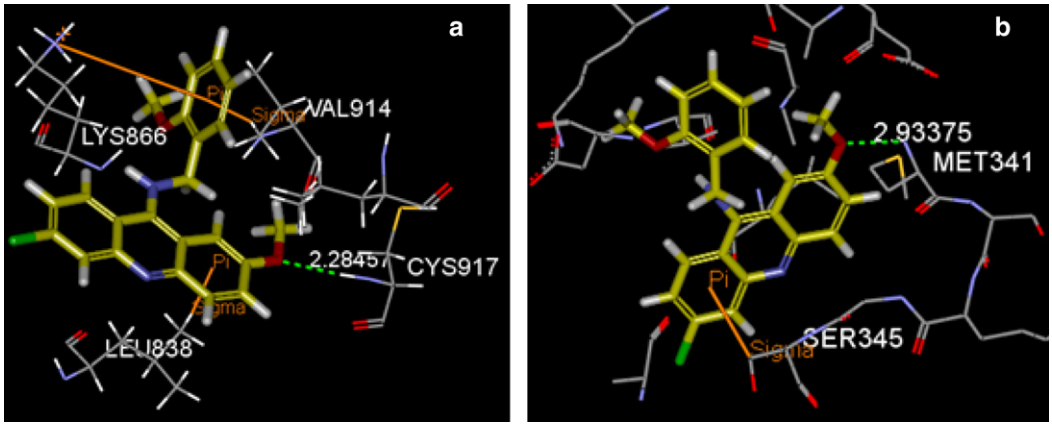


Figure 3. Docking of compound 7**r** on kinases. Molecules are colored by atom type and hydrogen bonds are represented by green dotted lines. (a: VEGFR-2, PDB code 1YWN; b, Src, PDB code 2H8H).

Table 4
Estimated free energy of binding (kcal mol^{-1})

	Complex	Ligand	Receptor	ΔG
VEGFR-2	−16038.8	19.7339	−16025.0	−33.534
Src	−25554.0	19.6813	−25549.0	−24.281

indicated that the compound docked well and could interact with the key residues of the Src binding site. The methoxy group of **7r** could form a hydrogen bond with Met341 with distance of 2.934 Å, and the π–sigma interaction was also occurred between the phenyl group of **7r** with Ser345. The LibDockScore is 65.066 The molecular dynamics were also studied and the results indicated that compound **7r** binded with substantially less affinity to Src compared to VEGFR-2 (Table 4), which corresponded with the results of kinase inhibition assay in vitro.

The docking analysis indicated that compound **7r** could fit into the binding site of VEGFR-2 and Src kinases, which also indicated that this compound may be potent VEGFR-2 and Src inhibitor.

3. Conclusion

In conclusion a series of 9-aminoacridine derivatives have been discovered and synthesized as potent VEGFR-2 and Src inhibitors. Some compounds showed low μM antiproliferative activity against K562, HepG-2, and MCF-7 cells in vitro. The structure–activity relationship studies indicated that the moiety located at the C-9 position of the acridine ring contributed much to the cytotoxicity. The molecular docking studies as well as the results of kinase inhibition assay in vitro indicated that compound **7r** may be potent VEGFR-2 and Src inhibitor. Further optimization of the structure to improve VEGFR-2 and Src activity are ongoing. Our study suggested that the acridine scaffold, which has been historically used for deriving topoisomerase inhibitors, is a potentially interesting scaffold to derive novel multi-target kinase inhibitors such as VEGFR-2 and Src dual inhibitors without topoisomerase inhibitory activity. It is of interest to determine if derivatives with dual kinase and topoisomerase inhibitory activities may be derived from this scaffold.

4. Experimental section

4.1. Synthesis and characterization

See Supplementary data for general methods and the preparation of compounds **5**–**6**.

4.1.1. General procedure for compounds 1, 2, 7a–7n

2-Methoxy-6,9-dichloroacridine (**6**) (0.20 mmol) and aniline derivatives (0.25 mmol) were dissolved in ethanol and chloroform (4/1 v/v). After adding a few drops of concentrated hydrogen chloride, the mixture was stirred at room temperature overnight and evaporated under reduced pressure to give solid compounds. The products were purified by recrystallization from ethanol.

4.1.1.1. *N*-(3-(6-Chloro-2-methoxyacridin-9-ylamino)phenyl) acetamide (1). Yield 67%; mp 161–163 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H), 8.12–7.86 (m, 3H), 7.72–7.50 (m, 3H), 7.48–7.20 (m, 3H), 6.91–6.75 (m, 1H), 3.73 (s, 3H), 2.04 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.08, 156.12, 151.21, 143.74, 141.93, 141.23, 138.02, 130.34, 128.24, 127.70, 124.39, 120.16, 117.47, 116.36, 114.15, 113.28, 103.89, 56.12, 24.52; HRMS(ESI): Calcd for $C_{22}H_{19}ClN_3O_2$ $[M+H]^+$ 392.1166. Found: 392.1162.

4.1.1.2. 4-(6-Chloro-2-methoxyacridin-9-ylamino)-2-hydroxybenzoic acid (2). Yield 75%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.47 (s, 1H), 8.24 (s, 1H), 8.16 (d, J = 9.3 Hz, 1H), 8.06 (d, J = 9.3 Hz, 1H), 7.83 (s, 1H), 7.76–7.83 (m, 2H), 7.49–7.52 (m, 1H), 6.93 (s, 1H), 6.80–6.83 (m, 1H), 3.83 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.83, 162.71, 156.90, 152.32, 148.76, 140.36, 139.45, 137.14, 132.08, 129.63, 128.47, 125.57, 122.11, 119.07, 118.25, 114.62, 113.61, 110.20, 110.03, 103.69, 56.56; HRMS(ESI): Calcd for $C_{21}H_{16}ClN_2O_4$ $[M+H]^+$ 395.0799. Found: 395.0793.

4.1.1.3. 6-Chloro-2-methoxy-*N*-(4-methoxyphenyl)acridin-9-amine (7a). Yield 64%; mp 302–306 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ11.33 (s, 1H), 7.98–8.03 (m, 3H), 7.73 (d, J = 7.8 Hz, 2H), 7.40–7.45 (m, 3H), 7.12 (d, J = 8.7 Hz, 2H), 3.83 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.39, 155.51, 153.41, 139.75, 138.63, 135.57, 133.17, 128.00, 127.70, 126.35, 123.66, 120.82, 117.71, 115.07, 114.66, 111.28, 103.58, 55.70, 55.47; HRMS(ESI): Calcd for $C_{21}H_{18}ClN_2O_2$ $[M+H]^+$ 365.1057. Found: 365.1050.

4.1.1.4. 6-Chloro-2-methoxy-*N*-(3-methoxyphenyl)acridin-9-amine (7b). Yield 71%; mp 280–282 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s, 1H), 8.12 (s, 1H), 8.06 (d, J = 9.0 Hz, 2H), 7.75 (d, J = 7.0 Hz, 2H), 7.44 (dd, J = 16.6, 8.6 Hz, 2H), 7.06 (s, 1H), 6.99 (d, J = 8.0 Hz, 2H), 3.75–3.85 (m, 6H); ^{13}C NMR(100 MHz, DMSO-d_6) δ 160.31, 155.73, 153.23, 141.95, 139.73, 138.83, 135.83, 130.67, 128.47, 127.82, 124.06, 121.06, 117.92, 116.40, 115.40, 112.88, 112.00, 110.07, 103.35, 55.71, 55.40; HRMS(ESI): Calcd for $C_{21}H_{18}ClN_2O_2$ $[M+H]^+$ 365.1057. Found: 365.1053.

4.1.1.5. 6-Chloro-2-methoxy-*N*-(2-methoxyphenyl)acridin-9-amine (7c). Yield 82%; mp 279–281 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.18 (s, 1H), 8.10 (d, J = 2.1 Hz, 1H), 8.08 (d, J = 9.6 Hz, 1H), 8.03 (d, J = 9.3 Hz, 1H), 7.73 (dd, J = 9.3, 2.6 Hz, 1H), 7.69 (d, J = 2.2 Hz, 1H), 7.55 (dd, J = 7.7, 1.5 Hz, 1H), 7.53–7.46 (m, 1H), 7.43 (dd, J = 9.4, 2.2 Hz, 1H), 7.26 (dd, J = 8.4, 0.9 Hz, 1H), 7.18 (td, J = 7.6, 1.1 Hz, 1H), 3.69 (s, 3H), 3.58 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.66, 153.96, 153.37, 139.30, 138.86, 135.38, 129.50, 128.54, 128.22, 126.94, 126.72, 124.05, 121.42, 120.93, 117.79, 114.52, 112.88, 111.62, 102.82, 55.68, 55.55; HRMS(ESI): Calcd for $C_{21}H_{18}ClN_2O_2$ $[M+H]^+$ 365.1057. Found: 365.1045.

4.1.1.6. 6-Chloro-*N*-(2-ethoxyphenyl)-2-methoxyacridin-9-amine (7d). Yield 68%; mp 193–195 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.15 (s, 1H), 8.13 (d, J = 9.4 Hz, 1H), 7.99 (s, 1H), 7.95 (d, J = 9.3 Hz, 1H), 7.75 (dd, J = 9.3, 2.3 Hz, 1H), 7.67 (s, 1H), 7.61 (d, J = 6.9 Hz, 1H), 7.51–7.42 (m, 2H), 7.29–7.12 (m, 2H), 3.79 (q, J = 6.9 Hz, 2H), 3.68 (s, 3H), 0.68 (t, J = 6.9 Hz, 3H); HRMS(ESI): Calcd for $C_{22}H_{20}ClN_2O_2$ $[M+H]^+$ 379.1213. Found: 379.1213.

4.1.1.7. 6-Chloro-*N*-(4-ethoxyphenyl)-2-methoxyacridin-9-amine (7e). Yield 74%; mp 280–281 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 8.05 (d, J = 1.9 Hz, 1H), 8.01 (t, J = 9.4 Hz, 2H), 7.72 (d, J = 7.5 Hz, 2H), 7.42 (dd, J = 9.4, 2.0 Hz, 1H), 7.38 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.9 Hz, 2H), 4.09 (q, J = 6.9 Hz, 2H), 3.74 (s, 3H), 1.36 (t, J = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.49, 155.45, 153.07, 139.91, 138.43, 135.77, 133.29, 127.80, 127.68, 126.13, 123.58, 120.99, 117.88, 115.51, 114.77, 111.42, 103.57, 63.42, 55.67, 14.48; HRMS(ESI): Calcd for $C_{22}H_{20}ClN_2O_2$ $[M+H]^+$ 379.1213. Found: 379.1202.

4.1.1.8. 6-Chloro-2-methoxy-*N*-(3,4,5-trimethoxyphenyl)acridin-9-amine (7f). Yield 80%; mp 251–253 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s, 1H), 8.12 (s, 1H), 8.07 (t, J = 9.6 Hz, 2H), 7.74 (d, J = 9.2 Hz, 2H), 7.47 (d, J = 9.4 Hz, 1H), 6.85 (s, 2H), 3.77 (s, 3H), 3.72 (s, 3H), 3.71 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.56, 153.62, 153.18, 139.64, 138.65, 136.62, 136.36, 135.69, 128.19, 127.80, 123.80, 120.78, 117.70, 115.04, 111.75, 103.48, 102.77, 60.18, 56.23, 55.73, 40.13, 39.92, 39.71, 39.50, 39.29, 39.08, 38.88; HRMS(ESI): Calcd for $C_{23}H_{22}ClN_2O_4$ $[M+H]^+$ 426.1268. Found: 425.1267.

4.1.1.9. 3-(6-Chloro-2-methoxyacridin-9-ylamino)phenol (7g). Yield 71%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 1H), 9.95 (s, 1H), 8.09 (d, J = 9.4 Hz, 1H), 8.05 (d, J = 1.7 Hz, 1H), 8.00 (d, J = 9.3 Hz, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.47 (dd, J = 9.4, 1.9 Hz, 1H), 7.31 (t, J = 8.1 Hz, 1H), 6.90–6.68 (m, 3H), 3.75 (s, 3H); HRMS(ESI): Calcd for $C_{20}H_{16}ClN_2O_2$ $[M+H]^+$ 351.0900. Found: 351.0896.

4.1.1.10. 2-(6-Chloro-2-methoxyacridin-9-ylamino)phenol (7h). Yield 56%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.17 (s, 1H), 10.19 (s, 1H), 8.13 (d, J = 9.2 Hz, 1H), 8.07 (d, J = 2.1 Hz, 1H), 8.01 (d, J = 9.2 Hz, 1H), 7.75 (s, 1H), 7.72 (dd, J = 9.2, 2.5 Hz, 1H), 7.46 (m, 2H), 7.38–7.28 (m, 1H), 7.03–7.05 (m, 2H), 3.70 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.13, 154.71, 152.44, 139.85, 139.40, 135.81, 129.96, 128.66, 127.73, 127.67, 127.46, 124.48, 121.36, 120.61, 118.21, 117.39, 115.02, 112.14, 103.52, 56.09; HRMS(ESI): Calcd for $C_{20}H_{16}ClN_2O_2$ $[M+H]^+$ 351.0900. Found: 351.0905.

4.1.1.11. *N*1-(6-Chloro-2-methoxyacridin-9-yl)benzene-1,4-diamine (7i). Yield 63%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 1H), 8.04 (s, 2H), 7.97 (d, J = 9.2 Hz, 1H), 7.72 (s, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.36 (d, J = 7.7 Hz, 1H), 7.11 (d, J = 8.1 Hz, 2H), 6.71 (d, J = 8.2 Hz, 2H), 5.61 (s, 2H), 3.75 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.31, 153.20, 148.36, 139.93, 138.52, 135.42, 127.68, 127.59, 125.99, 123.35, 120.83, 117.72, 114.50, 111.17, 103.72, 55.59; HRMS(ESI): Calcd for $C_{20}H_{17}ClN_3O$ $[M+H]^+$ 350.1060. Found: 350.1052.

4.1.1.12. 6-Chloro-2-methoxy-*N*-*p*-tolylacridin-9-amine (7j). Yield 71%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (s, 1H), 8.09–7.96 (m, 3H), 7.73 (dd, J = 9.2, 2.5 Hz, 1H), 7.69 (s, 1H), 7.44 (dd, J = 9.4, 2.1 Hz, 1H), 7.30–7.43 (m, 4H), 3.72 (s, 3H), 2.38(s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.39, 151.38, 140.92, 139.68, 137.64, 137.24, 135.25, 130.09, 127.60, 127.18, 123.59, 123.04, 122.21, 119.07, 115.97, 112.83, 103.45, 55.53, 20.51; HRMS-(ESI): Calcd for $C_{21}H_{18}ClN_2O$ $[M+H]^+$ 349.1108. Found: 349.1105.

4.1.1.13. 6-Chloro-2-methoxy-*N*-(4-(trifluoromethyl)phenyl) acridin-9-amine (7k). Yield 66%; mp 287–289 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.36 (s, 1H), 8.19 (s, 1H), 8.11 (d, J = 9.4 Hz, 2H), 7.75–7.85 (m, 3H), 7.69 (s, 1H), 7.55 (d, J = 8.9 Hz, 1H), 7.45–7.53 (m, 2H), 3.76 (s, 3H); HRMS(ESI): Calcd for $C_{21}H_{15}ClF_3N_2O$ $[M+H]^+$ 403.0825. Found: 403.0817.

4.1.1.146-Chloro-2-methoxy-***N***-(4-(trifluoromethoxy)phenyl) acridin-9-amine (**7l**).

Yield 66%; mp 288–290 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (d, J = 9.1 Hz, 1H), 8.01 (s, 1H), 7.94 (d, J = 9.1 Hz, 1H), 7.57 (d, J = 8.1 Hz, 1H), 7.35–7.45(m, 4H), 7.25 (d, J = 7.9 Hz, 2H), 3.66 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.36, 149.63, 144.33, 141.82, 138.81, 137.01, 127.44, 126.71, 124.01, 123.91, 123.49, 122.53, 122.33, 121.37, 120.21, 118.83, 117.33, 116.29, 114.93, 103.42, 55.23; HRMS(ESI): Calcd for $C_{21}H_{15}ClF_3N_2O_2$ $[M+H]^+$ 419.0774. Found: 419.0772.

4.1.1.15. 6-Chloro-*N*-(2-fluorophenyl)-2-methoxyacridin-9-amine (7m). Yield 69%; mp 280–282 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 8.15 (s, 1H), 8.13–8.04 (m, 2H), 7.76 (d, J = 9.3 Hz, 1H), 7.71 (s, 1H), 7.65 (t, J = 7.5 Hz, 1H), 7.50 (d, J = 9.4 Hz, 2H), 7.43 (dd, J = 14.4, 7.1 Hz, 2H), 3.72 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.45, 156.55, 154.98, 154.08, 139.94, 139.56, 136.30, 129.81, 129.74, 129.20, 128.03, 127.28, 126.40, 126.37, 125.22, 121.68, 118.58, 117.53, 117.33, 115.75, 112.64, 103.31, 56.30; HRMS(ESI): Calcd for $C_{20}H_{15}ClFN_2O$ $[M+H]^+$ 353.0857. Found: 353.0847.

4.1.1.16. 6-Chloro-*N*-(2-chlorophenyl)-2-methoxyacridin-9-amine (7n). Yield 62%; mp 287–289 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.44 (s, 1H), 8.12 (d, J = 14.4 Hz, 1H), 8.07 (d, J = 8.8 Hz, 1H), 8.00 (d, J = 9.3 Hz, 1H), 7.74 (d, J = 8.0 Hz, 2H), 7.67 (dd, J = 6.4 Hz 1H), 7.56–7.60 (m, 2H), 7.46–7.54 (m, 2H), 3.65 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.60, 153.00, 139.42, 138.59, 135.50, 130.51, 128.87, 128.74, 127.68, 127.52, 126.83, 124.11, 120.78, 117.68, 115.14, 112.27, 103.18, 55.55; HRMS(ESI): Calcd for $C_{20}H_{15}Cl_2N_2O$ $[M+H]^+$ 369.0561. Found: 369.0550.

4.1.2. General procedure for compound 7o–7r

Various anilines/amines (2.50 mmol) were dissolved in dry DMF (15 mL) and then sodium hydride (2.50 mmol) was added. The mixture was stirred under argon for 1 h at room temperature. The compound **6** (1.00 mmol) and potassium iodide (0.25 mmol) were added. The mixture was stirred overnight and then poured into water (50 mL), extracted with ethyl acetate to give the crude product which had been purified by column chromatography using petroleum ether and ethyl acetate (3:1 v/v) as the eluent.

4.1.2.1. 6-Chloro-2-methoxy-*N*-(pyridin-4-yl)acridin-9-amine (7o). Yield 56%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.65 (s, 1H), 8.36–7.82 (m, 5H), 7.55 (s, 2H), 7.27 (s, 1H), 6.62 (s,

2H), 3.78 (s, 3H); HRMS (ESI): Calcd for $C_{19}H_{15}ClN_3O$ $[M+H]^+$ 336.0904. Found: 336.0904.

4.1.2.2. *N*3-(6-Chloro-2-methoxyacridin-9-yl)pyridine-2,3-diamine (7p). Yield 60.4%; mp 159–161 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 7.96 (s, 1H), 7.68 (d, J = 4.0 Hz, 1H), 7.60–7.16 (m, 4H), 7.06 (s, 1H), 6.68 (m, 1H), 6.59–6.32 (m, 1H), 5.65 (s, 2H), 3.59 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.90, 170.21, 153.80, 151.48, 150.26, 142.12, 140.91, 135.07, 134.50, 131.95, 128.30, 123.55, 122.34, 120.87, 119.86, 118.09, 112.59, 106.16, 54.92; HRMS (ESI): Calcd for $C_{19}H_{16}ClN_4O$ $[M+H]^+$ 351.1013. Found: 351.1016.

4.1.2.3. *N*2-(6-Chloro-2-methoxyacridin-9-yl)pyridine-2,6-diamine (7q). Yield 68%; mp 224–226 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.31 (s, 1H), 8.11–7.84 (m, 3H), 7.48 (d, J = 9.0 Hz, 1H), 7.40 (s, 2H), 7.25 (t, J = 7.7 Hz, 1H), 5.94 (t, J = 10.8 Hz, 1H), 5.85 (dd, J = 31.1, 7.9 Hz, 1H), 5.63 (s, 2H), 3.81 (s, 3H); HRMS(ESI): Calcd for $C_{19}H_{16}ClN_4O$ $[M+H]^+$ 351.1013. Found: 351.1012.

4.1.2.4. 6-Chloro-2-methoxy-*N*-(2-methoxybenzyl)acridin-9-amine (7r). Yield 81%; mp 144–146 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, J = 9.3 Hz, 1H), 7.88 (s, 1H), 7.82 (d, J = 8.8 Hz, 1H), 7.52 (d, J = 6.6 Hz, 1H), 7.48 (d, J = 1.7 Hz, 1H), 7.37 (dd, J = 9.2, 1.7 Hz, 1H), 7.29 (t, J = 7.0 Hz, 2H), 7.02 (d, J = 8.1 Hz, 1H), 6.96 (t, J = 7.4 Hz, 1H), 4.87 (s, 2H), 3.73 (s, 3H), 3.65 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.32, 154.73, 150.42, 147.87, 146.21, 133.33, 130.64, 128.40, 127.75, 127.42, 127.11, 125.82, 124.18, 122.71, 120.27, 116.60, 114.52, 110.48, 100.67, 55.18, 54.98, 47.42; HRMS (ESI): Calcd for $C_{22}H_{20}ClN_2O_2$ $[M+H]^+$ 379.1213. Found: 379.1203.

4.1.2.5. *N*1-(6-Chloro-2-methoxyacridin-9-yl)ethane-1,2-diamine hydrochloride (7s). Yield 58%; mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (d, J = 9.2 Hz, 1H), 7.92–7.78 (m, 2H), 7.64 (d, J = 2.3 Hz, 1H), 7.41 (dd, J = 9.3, 2.1 Hz, 1H), 7.32 (d, J = 9.1 Hz, 1H), 3.93 (s, 3H), 3.73 (t, J = 6.3 Hz, 2H), 2.88 (t, J = 6.3 Hz, 2H); HRMS(ESI): Calcd for $C_{16}H_{17}Cl_2N_3O$ $[M-Cl]^+$ 302.1060. Found: 302.1058.

4.2. Molecular simulation

Docking studies of the representative compound **7r** were performed with the molecular modeling package Discovery Studio.2.5/Libdock protocol (*Accelrys Software Inc.*). The protein's three dimensional structure from the Brookhaven Protein Data Bank (PDB) were used as raw models for the docking studies, VEGFR-2 (PDB code 1YWN)[40] and Src (PDB code 2H8H).[41] Hydrogen atoms were added and water molecules co-crystallized with the protein were removed from the original structure. The general procedure is as followed: (1) ligand and receptor preparation, (2) protocol generation, (3) docking and scoring and (4) analysis of the results.

The Discovery Studio 2.5/Standard Dynamics Cascade protocol (*Accelrys Software Inc.*) was used for molecular dynamics (MD) simulations. The general procedure for MD is as followed: (1) preparing ligand–receptor complex; (2) performing MD simulation, (3) drawing step–potential energy curve and recording the lowest energy conformation and (4) calculating binding energy by MM/PBSA protocol

4.3. Biological activity

4.3.1. Cell growth inhibition assay

K562 (suspension cells line) was cultured in RPMI-1640, HepG-2 and MCF-7 (adherent cell lines) was cultured in DMEM, with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin in humidified air at 37 °C with 5% CO_2. Viable cell were seeded into 96-well tissue plate at 1.5×10^5 cells/mL, treated with the synthesized compounds at various concentrations. After 48 h treatment, the cells were incubated with 15 μL MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide from Sigma) solution (5 mg/mL) for 4 h at 37 °C, 5% CO_2. The formazan precipitates were dissolved in 100 μL DMSO and the absorbance at 495 nm of each well was measured by Multimode Detector DTX 880 (Beckman Coulter).[42]

4.3.2. In vitro kinase assays on VEGFR-2 and Src

In vitro kinase assays on VEGFR-2 and Src inhibition were tested by HD Biosciences Co., Ltd in Shanghai, China. General procedures are as the following: Kinases were incubated with substrates, compounds and ATP in a final buffer of 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.01% Triton X-100, 100 μg/mL BSA, 2.5 mM DTT in 384-well plate with the total volume of 10 μL. The assay plate was incubated at 30 °C for 1 h and stopped with the addition of equal volume of kinase-glo plus reagent. The luminescence was read at envision. The signal was correlated with the amount of ATP present in the reaction and was inversely correlated with the kinase activity.[43]

4.3.3. Western blot analysis

K562 cells were cultured in 2 cm dishes for 0, 1, 2, 4 h, followed by treatment with compound **7r** (10 μM) for different time-periods. Cells were centrifuged and treated with lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 1 mM EDTA; 0.1% NP-40; 0.5 mM Na_3VO_4; 1 mM NaF; and protease inhibitor cocktail (Thermo scientific)) on ice for 0.5 h, followed by centrifugation at 14,000 rpm for 10 min. Protein concentrations in the supernatant were determined using Coomassie brilliant blue, as described in the manufacturer's manual. Lysate proteins were subjected to 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to PVDF membrane (amc Biobind NT-200). After blotting, the membrane was blocked in 5% BSA (TBS, 0.5 mM Na_3VO_4; 1 mM NaF) for 1 h, and incubated with the specific primary antibody for 2 h at room temperature (ERK1/2 (Bioworld Cat. No. BS3628), p-ERK1/2 (Bioworld Cat. No. BS5016)). Protein bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo scientific) after hybridization with the HRP-conjugated secondary antibody.

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Supplementary data

Supplementary data (general methods and the preparation of compound **5**, **6**; Human Topoisomerase II Assay; ^{1}H NMR and ^{13}C NMR spectrum and High resolution mass spectrometry) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.053.

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