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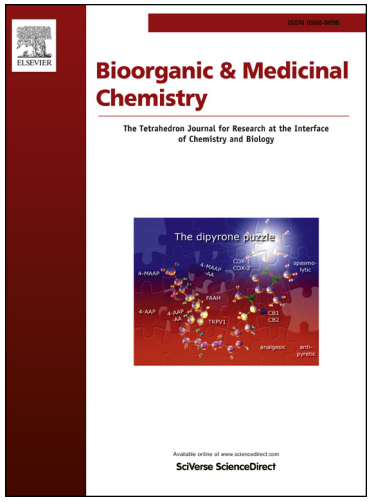

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Design, Synthesis and Evaluation of Acridine Derivatives as Multi-Target Src and MEK Kinase Inhibitors for Anti-tumor Treatment

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Abstract

Clinical studies have shown enhanced anticancer effects of combined inhibition of Src and MEK kinases. Development of multi-target drugs against Src and MEK is of potential therapeutic advantage against cancers. As a follow-up of our previous studies, and by using molecular docking method, we designed and synthesized a new series of 9-anilinoacridines containing phenyl-urea moieties as potential novel dual Src and MEK inhibitors. The anti-proliferative assays against K562 and HepG-2 tumor cells showed that most of the derivatives displayed good cytotoxicy *in vitro*. In particular, kinase inhibition assays showed that compound **8m** inhibited Src (59.67%) and MEK (43.23%) at 10 μM, and displayed moderate inhibitory activity against ERK and AKT, the downstream effectors of both Src and MEK. Moreover, compound **8m** was found to induce K562 cells apoptosis. Structure−activity relationships of these derivatives were analyzed. Our study suggested that acridine scaffold, particularly compound **8m**, is of potential interest for developing novel multi-target Src and MEK kinase inhibitors.

Key words: Src; MEK; kinase inhibitor; acridine; antitumor; apoptosis

1. Introduction

Src is one of non-receptor type tyrosine kinases, which was first identified as a protein encoded by sarcoma sirus,[1] and subsequently found to be a critical regulator of a large number of intracellular signaling pathways.[2] Recent studies have shown that dysregulation of Src is strongly associated with malignant progression of human tumors.[3] As Src was wildly expressed in tumor cells, and controlled a myriad of cellular processes including proliferation, migration, invasion, gene transcription, differentiation, angiogenesis, and survival,[4] it has been pursued as a potential drug target for cancer and related bone disease.[5] Development of Src inhibitors has become a main focus in antitumor drug campaigns.[2c, 5b, 6] At present, a series of Src inhibitors have been developed, some of which have entered into clinical research phases, such as Dasatinib,[7] Bosutinib,[8] Ponatinib,[9] and Saracatinib, et al.[2d, 10]

Although Src inhibitor drugs have exhibited highly promising anticancer effects, much work needs to be done since most of the drugs suffered from low tumor response or resistance.[11] The resistance to Src inhibitors may partly arise from the crosstalk between Src-pathway and other pathways.[12] Therefore, the exploration of multi-target drugs or drug combinations to simultaneously inhibit the drug target and resistant pathways can provide a more effective approach for cancer.[13] Recent study indicated that combined inhibition of Src and MEK was a promising approach to improve the efficacy of cancer therapy.[14] MEK kinase itself is an important anticancer target [15] because of its key roles in regulating mitogen-activated protein kinase (MAPK) signaling pathway in apoptosis and oncogenic transformation.[16] However, only 22% patients with BRAF mutations are sensitive to MEK inhibitor (Trametinib) in a clinical trial for melanoma.[17] Combinatorial approaches are necessary to improve the clinical response.[18] As Src and MEK inhibitors displayed synergistic effects[14a], development of multi-target drugs targeted both Src and MEK may therefore

offer a better therapeutic advantage.

Acridine derivatives have been explored as potential therapeutic agents for cancer treatment[19] particularly for targeting DNA and DNA related enzymes, such as topoisomerases, telomerase, et al.[20] However, some acridine derivatives have been found to inhibit the activity of tyrosine kinases.[21] In our pursuit of novel acridine analogues with antitumor activity,[22] through using molecular docking and SVM high throughput virtual screening methods, we found that the acridine derivative **OA** (Figure 1) inhibited Src activity.[23] However, the inhibitory activity is very weak likely due in part to only one hydrogen bond formed between the acridine derivative and the hinge of Src protein. Shokat et al. had found that small molecules bearing a moity such as urea bonds with the ability to bind to the DFG-out conformation of c-Src could improve the kinase inhibition affinity greatly.[24] In addition, some kinase drugs approved or in clinical trails, such as Imatinib,[25] Ponatinib,[26] and Regorafenib,[27] all contain amido or urea moieties, which contribute much to the kinase binding affinity due to that they can form hydrogen bonds with the amino-acid residues of the DFG-out pocket of kinases.[28]

Based on the above considerations and the characteristics of protein kinase inhibitors with the current state of Src and MEK kinase inhibitors, we introduced the urea moities to the 9-anilinoacridine substituents (Table 1) such as compound **8a** (Figure 2a) to improve the bioactivity. In order to further understand the interaction between compound **8a** and kinases, molecular docking studies were performed using the Discovery Studio 3.1. Figure 2b and 2c indicated that compound **8a** could dock into ATP binding sites of both Src and MEK. For Src, nitro group of compound **8a** could form a hydrogen bond with Met341 in the hinge region. Meanwhile, as we expected, -NH of the urea moiety formed a hydrogen bond with Glu310 in the DFG-out pocket. In addition, π-π interaction was formed between the acridine ring with Phe405. Cation–π interaction was also

occurred between the phenyl group and Tyr340. For MEK, the nitro group of compound **8a** enabled the formation of hydrogen bonds with Asn221 and Arg234 in the hinge region of MEK. -NH of the urea linkage formed two hydrogen bonds with Phe209 in the DFG-out region. All the results revealed that compound **8a** could be a potential dual inhibitor of Src and MEK kinases.

In this article, a series of compound **8a** derivatives with different substitents at acridine and phenyl rings were designed and synthesized. Preliminary *in vitro* anti-proliferative ability of synthetic compounds against related human cancer cell-lines (K562 and HepG-2 cells) were studied, and structure–activity relationship was disclosed. Inhibition rates of the representative synthetic compounds against Src and MEK kinase were also evaluated.

2. Results and discussions

2.1 Chemistry

The synthetic pathway was illustrated in Scheme 1. The reaction of 2-chlorobenzoic acid derivatives **1** with the anilines **2** in the presence of Cu afforded the corresponding anthranilic acids **3** (Ullmann conditions).[29] Cyclization of compound **3** using $POCl_3$ gave the 9-chloroacridine derivatives **4 (4a-4g)** by using column chromatography (petroleum ether: ethyl acetate=20:1). Compound **5** was reacted with anilines bearing different substituents to give compound **6 (6a-6l)**. Subsequent reduction of **6 (6a-6l)** in the presence of Fe and NH_4Cl led to the expected phenyl-urea derivatives **7 (7a-7l)**.[30] Compound **7 (7a-7l)** (0.22 mmol) in THF (10 mL) which then underwent nucleophilic substitution reaction with compound **4 (4a-4g)** (0.20 mmol) in the presence of three drops of concentrated hydrogen chloride.The mixture was stirred for two days at room temperature. The raw products were filtered off, washed with diethyl ether. Finally, the raw products were filtered off, washed with diethyl ether to obtain the desired compound **8 (8a-8r)** in moderate to

good yields.

2.2 *In vitro* cell growth inhibition assay

The antiproliferative activity of the desired acridine derivatives bearing phenyl-urea group at 9-position was tested against K562 and HepG-2 cells by MTT assay, and Imatinib was used as the positive control. As shown in Table 2, most of the acridine derivatives displayed good antiproliferative activity with low micromolar IC_{50} values, and both the substituents in the acridine ring (A) and phenyl ring (B) affected the antiproliferative activity significantly. As synthesized compounds were more sensitive to K562 cells than HepG-2 cells, K562 cells were selected to study the structure-activity relationship.

2.2.1. Substitution Effects of the benzene ring A (R_5, R_6, R_7)

As literature reported, the substituents in phenyl ring A played an important role in the bioactivity.[31] A series of compounds **8** (**8a−8l)** bearing the same acridine core but with different substituents atR_5, R_6, R_7 were synthesized (Table 2). For R_5, the introduction of methoxyl group had almostly no effect on the cytotoxicity (**8a** vs. **8b**). For R_6, compound **8c** with methoxyl group dispalyed similar IC_{50} value compared with **8a**. By changing methoxyl (**8c**) to methyl (**8h**) or ethyl (**8i**) group, compounds showed decreasing inhibitory effect. However, introduction of tri-fluoromethyl substituent showed much stronger inhibition effect, approximately 3-fold more potent than **8a**. For R_7, a different trend could be seen compared to that for R_6. For example, when methxoyl group was introduced, compound **8d** displayed an apparent reduced activity. However, introduction of alkyl group (**8j**:ethyl; **8k**:propyl; **8l**:butyl) led to an increased antiproliferative activity compared to **8a**. These results indicated that the position, electron-negativity and steric effect in the benzene ring may change the cytotoxic profile.

2.2.2. Substitution Effects of the ring of acridine (ring B)

Molecular modeling results (Figure 2, Figure 1s and Table 1s) indicated that nitro or methoxyl group at R_1 position could form hydrogen bonds to the key residues of Src and MEK. Therefore, most of the acridine compounds were modified with these moieties, such as **8a-8m, 8o-8p**. As seen

from Table 1, the antiproliferative activity of compound **8m** with methoxyl group was comparable to that of **8a** with nitro group. The results indicated that the R_1 position might tolerate both nitro and methoxyl group, which was in agreement with molecular docking results. When methoxyl group was introduced to the R_3 or R_4 position, almost no distinct effect on the bioactivity was observed, such as **8o-8q**. To our surprise, compound **8n** with no substitents at R_1 position and methoxyl group at R_2 position displayed the best antiproliferative activity with an IC_{50} value at 0.49 μM. As for B ring, the introduction of tri-fluoromethyl group at R_6 position **(8g)** could improve the cytotoxity, compound **8r** containing both substituents of **8n** and **8g** was synthesized to increase the antiproliferative activity. To our disappointment, this modification led to approximately 4-fold less activity than that of compound **8n**.

2.3 *In vitro* kinase inhibition assay

As compounds **8m**, **8n** and **8q** had good bioactivities against both K562 and HepG-2 cells with IC_{50} values all below10 μM, they were firstly selected to screen *in vitro* kinase inhibition assay to evaluate whether they can truly inhibit Src activity. Unfortunately, only compound **8m** potently inhibited 59.67% of Src activity at 10 μM. At the same condition, compound **8m** exhibited moderate activity against MEK and PI3K with inhibition rates of 43.23% and 44.41%, respectively. Although the kinases activities of compound **8m** were weaker than the antiproliferative activity, the synergistic effects of multi-target agents of moderate activities might contribute to the cytotoxicity.[32]

Furthermore, the influences of compound **8m** on the protein expression levels of ERK and AKT, the downstream kinases of Src, MEK and PI3K were evaluated. K562 cells were treated with compound **8m** at different concentrations (0, 0.5, 2.5, 5, and 10 μM) for 48 h. The results (Figure 3) indicated that **8m** reduced the expression of ERK and AKT in a dose-dependent manner. In addition, DNA binding ability of **8m** was evaluated by UV-vis spectrophotometric titration test. The binding constant was 0.083 M^{-1}, which was much lower than DNA binding agents (Figure 2s, supporting data). Therefore, DNA binding ability was not contributed to the antiproliferative activity of

compound **8m**. All the results suggested that the inhibition of Src, MEK and PI3K might mainly contribute to the good activities of compound **8m** against K562 and HepG-2 cell lines.

2.4 Effects of Compound 8m on Cell Apoptosis

Literature reported that inhibition Src activity can induce cancer cell apoptosis, such as the preclinical drugs AZM475271,[33] SU6656,[34] etc.[35] Compound **8m** with the best kinases inhibition rates was selected to test its impact on apoptosis. As shown in Figure 4, compound **8m** induced apoptosis in K562 cells in a concentration-dependent manner. At the concentration increased from 0 μM to 10 μM, the early apoptosis ratio increased from 3.14% to 54.98%, when K562 cells were treated with compound **8m** for 48 h, which demonstrated that compound **8m** could effectively induce K562 cells apoptosis.

In addition, the expression of cleaved-caspase-3 and cleaved-caspase-7 were evaluated to further evaluate the apoptosis induced. As we know, caspases play an important role in apoptosis, among which caspase-3 and caspase-7 are executioner caspases. They will be cleaved by an initiator caspase after apoptotic signaling events occurred. Figure 5 showed that compound **8m** could induce significant activation of cleaved-caspase-3 and cleaved-caspase-7 at 10 μM for 48 h, suggesting the capability of **8m** to induce apoptosis. Moreover, PARP (poly ADP-ribose polymerase) can be activated in cells experiencing DNA damage to repair them, which become inactivated by caspase cleavage. Caspase-3 and caspase-7 are responsible for PARP cleavage. Therefore, we tested the induction of PARP cleavage by compound **8m**. The results in Figure 5 revealed the generation of the cleaved PARP. The results above suggested that compound **8m** could induce K562 cells apoptosis.

3. Conclusion

In this study, a series of novel multi-target inhibitors of Src and MEK bearing acridine and phenyl-urea scaffolds were designed and synthesized based on rational design and molecular docking methods. Most of these compounds displayed low micromolar IC_{50} values against K562

cells *in vitro*. Structure−activity relationship indicated that substitutions of the benzene ring (A) and acridine ring (B) play important roles in the antiproliferative activities of the tested compounds. Kinase assay study confirmed that compound **8m** is a novel multi-target Src and MEK kinases inhibitor. Moreover, compound **8m** was found to induce cancer cell apoptosis. Further optimization of this structure for improved anti-proliferative activity is ongoing. Our study suggested the potential use of the acridine compounds as multi-target kinase inhibitors, and indicated new possibilities for expanding the application of acridine compounds beyond their conventional DNA and DNA related enzyme targets.

4. Experimental section

4.1. Synthesis and characterization

See supporting information for synthetic methods and the preparation of compounds **4a-4g** and **7a-7l**.

4.1.1. General procedure for compounds 8 (8a-8r)

Compound **7** (**7a-7l)** (0.22 mmol) in THF (10 mL) was added three drops HCl (6 mM). The mixture was stirred for 0.5 h and then compound **4** (**4a-4g)** was added respectively (0.20 mmol).The mixture was stirred for two days at room temperature under nitrogen until TLC indicated the completion of reaction. The products were filtered off, washed with diethyl ether, and the pure target compounds **8** (**8a-8q**) were obtained.

4.1.1.1. 1-(4-((3-Nitroacridin-9-yl)methyl)phenyl)-3-phenyl)urea (8a). Yield 73%; red solid, mp 268.2-267.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (s, 1H), 9.12 (s, 1H), 8.68 (s, 1H), 8.43 (s, 1H), 8.42-8.41(m, 1H), 8.12 (s, 1H), 8.04 (d, J = 8.8 Hz, 1H), 7.94-7.90 (m, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 7.6 Hz, 2H), 7.42 (d, J = 8.8 Hz, 1H), 7.29 (dd, J = 8.0 Hz, 7.6 Hz, 4H), 6.98 (dd, J = 8.0Hz, 7.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 156.29, 153.37, 149.68, 145.80, 144.90, 141.60, 133.33, 130.48, 130.29, 130.16, 129.85, 129.01, 128.62, 126.04, 123.43, 123.28, 122.01,

121.65, 121.54, 119.85, 118.05, 118.01, 117.03, 114.63, 114.39, 114.34.HRMS (ESI) m/z calculated for $[M+H]^+$ 450.1566, found 450.1544.

4.1.1.2. 1-(2-Methoxyphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8b). Yield 75%; red solid, mp 232.8-233.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.74(s, 1H), 9.73 (s, 1H), 9.58, (s, 1H), 8.96 (s, 1H), 8.38 (s, 1H), 7.92 (s, 1H), 7.53-7.47 (m, 5H), 7.32 (s, 1H), 7.28 (m, 3H), 7.16 (s, 1H), 7.03 (s, 1H), 6.95 (s, 1H), 4.07 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.29, 153.28, 153.02, 149.68, 148.15, 145.78, 144.45, 133.32, 129.35, 129.0, 126.05, 123.28, 122.12, 121.75, 121.55, 121.20, 121.07, 120.95, 119.50, 119.27, 118.79, 118.63, 117.03, 114.70, 111.28. HRMS (ESI) m/z calculated for $[M+H]^+$ 480.1672, found 480.1667.

4.1.1.3. 1-(3-Methoxyphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8c). Yield 80%; red solid, mp 274.1-275.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.32 (s, 1H), 9.13 (s, 1H), 8.66 (s, 1H), 8.42 (s, 1H), 8.41 (s, 1H), 8.10 (s, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.94-7.88 (m, 2H), 7.59 (d, J = 8.8 Hz, 2H), 7.40(s, 1H), 7.26 (d, J = 7.6 Hz, 2H), 7.21 (s, 1H), 7.17 (d, J = 8.0 Hz, 1H), 6.94 (dd, J = 8.0 Hz, 8.0 Hz, 1H), 6.56 (dd, J = 8.0 Hz, 8.0 Hz, 1H), 3.73 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.61, 153.75, 153.15, 149.86, 140.42, 140.24, 140.18, 139.05, 137.02, 134.12, 129.39, 129.29, 128.77, 126.10, 125.19, 124.27, 122.35, 121.64, 119.28, 119.14, 118.65, 118.56, 116.55, 115.95, 115.59, 104.10, 56.40. HRMS (ESI) m/z calculated for $[M+H]^+$ 480.1672, found 480.1661.

4.1.1.4. 1-(4-Methoxyphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8d). Yield 53%; red solid, mp 283.1-284.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.32 (s, 1H), 8.95, (s, 1H), 8.76 (s, 1H), 8.45 (d, J = 4.8 Hz, 1H), 8.19 (s, 1H), 8.08 (d, J = 9.2 Hz, 1H), 7.98 (s, 2H), 7.62 (d, J = 8.8 Hz, 2H), 7.46 (s, 1H), 7.38-7.33 (m, 3H), 6.88 (d, J = 9.2 Hz, 2H), 3.72 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.07, 153.25, 150.41, 141.52, 140.19, 136.18, 133.13, 128.97, 126.52, 125.25,

124.65, 120.49, 119.74, 119.29, 116.47, 115.14, 114.54, 55.70. HRMS (ESI) m/z calculated for $[M+H]^+$ 480.1672, found 480.1661.

4.1.1.5. 1-(3-Fluorophenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8e). Yield 71%; red solid, mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.49 (s, 2H), 8.68, (s, 1H), 8.41 (d, J = 8.0 Hz, 1H), 8.11 (s, 1H), 8.02 (d, J = 9.2 Hz, 1H), 7.95-7.88 (m, 2H), 7.60 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 11.6 Hz, 1H), 7.38 (s, 1H), 7.34-7.28 (m, 3H), 7.12 (d, J = 8.0 Hz, 1H), 6.81-6.76 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.09, 161.70, 154.61, 152.95, 150.20, 142.10, 141.99, 141.61, 140.38, 139.06, 135.72, 130.84, 130.75, 128.94, 126.61, 124.53, 124.32, 119.71, 118.16, 116.22, 115.02, 114.31, 114.29, 108.73, 108.52, 105.34, 105.08. HRMS (ESI) m/z calculated for $[M+H]^+$ 468.1472, found 468.1490.

4.1.1.6. 1-(3-Chlorophenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8f). Yield 59%; red solid, mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.52 (s, 2H), 8.67, (s, 1H), 8.39 (s, 1H), 8.10 (s, 1H), 8.01 (d, J = 9.2 Hz, 1H), 7.91 (d, J = 10.8 Hz, 2H), 7.69 (s, 1H), 7.60 (d, J = 8.8 Hz, 2H), 7.38 (s, 1H), 7.30-7.28 (m, 4H), 7.01 (d, J = 5.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.62, 152.94, 150.20, 141.73, 141.58, 140.34, 139.13, 135.75, 133.70, 128.93, 126.59, 124.60, 124.35, 121.92, 119.70, 117.87, 116.94, 116.23, 115.02.HRMS (ESI) m/z calculated for $[M+H]^+$ 484.1176, found 484.1145.

4.1.1.7.1-(4-((3-Nitroacridin-9-yl)amino)phenyl)-3-(3(trifluoromethyl)phenyl)urea (8g). Yield 81%; red solid, mp 268.7-270.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.95(s, 1H),9.75 (s, 1H), 9.68 (s, 1H), 8.80 (s, 1H), 8.47 (d, J = 8.4 Hz, 1H), 8.22 (s, 1H), 8.10 (d, J = 9.6 Hz, 1H), 8.01 (s, 3H), 7.65 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.4 Hz, 1H), 7.54-7.49 (m, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 7.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.59, 153.08, 150.50, 141.46, 141.02, 140.18, 139.87, 136.43, 130.45, 130.23, 129.91, 128.96, 126.44, 126.05, 125.66, 124.86, 123.34,

122.15, 119.73, 118.63, 117.06, 116.62, 115.19, 114.47, 114.43, 65.38. HRMS (ESI) m/z calculated for $[M+H]^+$ 518.1440, found 518.1450.

4.1.1.8. 1-(4-((3-Nitroacridin-9-yl)amino)phenyl)-3-(m-tolyl)urea (8h). Yield 63%; red solid, mp 277.8-278.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.47 (s, 1H), 9.16, (s, 1H), 8.70 (s, 1H), 8.41 (s, 1H), 8.11 (s, 1H), 8.03 (d, J = 6.8 Hz, 1H), 7.91 (s, 2H), 7.60 (d, J = 7.6 Hz, 2H), 7.38 (s, 1H), 7.34-7.28 (m, 4H), 7.16 (d, J = 6.8 Hz, 1H), 6.78 (d, J = 6.4 Hz, 1H), 2.27 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.57, 153.00, 150.47, 141.49, 140.19, 140.14, 137.86, 136.40, 129.03, 126.47, 125.79, 124.87, 124.07, 120.75, 119.72, 119.40, 119.28, 116.94, 116.58, 115.18, 114.47, 25.64. HRMS (ESI) m/z calculated for $[M+H]^+$464.1723, found464.1729.

4.1.1.9. 1-(3-Ethylphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8i). Yield 47%; red solid, mp 275.4-275.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.37 (s, 1H), 9.10 (s, 1H), 8.65 (s, 1H), 8.39 (d, J = 8.8 Hz, 1H), 8.2 (s, 1H), 8.00 (d, J = 9.2 Hz, 1H), 7.91-7.86 (m, 2H), 7.59 (d, J = 8.8 Hz, 2H), 7.35 (s, 1H), 7.31 (s, 1H), 7.28 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 7.18 (m, 1H), 6.82 (d, J = 7.2 Hz, 1H), 2.57 (q, J = 7.6 Hz, 2H), 1.18 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 154.27, 153.11, 150.13, 144.82, 141.69, 140.49, 140.16, 139.16, 135.46, 129.16, 128.95, 126.68, 124.14, 123.81, 121.87, 119.70, 119.58, 118.16, 117.98, 116.08, 115.29, 114.98, 28.78, 16.00. HRMS (ESI) m/z calculated for $[M+H]^+$ 478.1879, found 478.1873.

4.1.1.10. 1-(4-Ethylphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8j). Yield 43%; red solid, mp 253.2-254.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.50 (s, 1H), 9.14 (s, 1H), 8.82 (s, 1H), 8.47 (s, 1H), 8.22 (s, 1H), 8.10 (d, J = 9.6 Hz, 1H), 8.02 (d, J = 7.6 Hz, 2H), 7.63 (d, J = 8.8 Hz, 2H), 7.55 (d, J = 8.8 Hz, 1H), 7.40-7.36 (m, 4H), 7.12 (d, J = 8.4 Hz, 2H), 2.52 (m, 2H), 1.16 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.60, 153.12, 150.49, 141.44, 140.44, 140.18, 137.81, 137.72, 136.44, 128.97, 128.47, 126.43, 125.71, 124.86, 123.96, 119.73, 119.35, 118.82,

116.60, 115.18, 28.00, 16.22. HRMS (ESI) m/z calculated for $[M+H]^+$ 478.1879, found 478.1895.

4.1.1.11. 1-(4-((3-Nitroacridin-9-yl)amino)phenyl)-3-(4-propylphenyl)urea (8k). Yield 80%; red solid, mp 271.0-272.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.48 (s, 1H), 9.13 (s, 1H), 8.81 (s, 1H), 8.48(s, 1H), 8.21 (s, 1H), 8.10 (d, J = 1.2 Hz, 1H), 7.62 (d, J = 8.4 Hz, 1H), 7.53 (d, J =8.4 Hz, 1H), 7.45 (s, 1H), 7.37(d, J = 8.4 Hz,2H), 7.25 (s, 1H), 7.22 (d, J = 9.6 Hz, 1H),7.10 (d, J = 8.4 Hz, 1H), 7.08 (s, 1H), 1.58-1.54(q, J = 7.6 Hz, 2H), 0.89(t, J = 7.6 Hz, 3H) ; ^{13}C NMR (100 MHz, DMSO-d_6) δ153.25, 137.44, 135.11, 129.12, 129.05, 126.57, 123.36, 122.94, 119.75, 119.11, 118.20, 114.94, 113.80, 37.03, 24.60,14.01.HRMS (ESI) m/z calculated for $[M+H]^+$ 492.2036, found492.2032

4.1.1.12. 1-(4-Butylphenyl)-3-(4-((3-nitroacridin-9-yl)amino)phenyl)urea (8l). Yield 44%; red solid, mp 279.4-281.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.43 (s, 1H), 9.08 (s, 1H), 8.79 (s, 1H), 8.46 (s, 1H), 8.22 (s, 1H), 8.10 (d, J = 8.8 Hz, 1H), 8.04-8.01 (m, 2H), 7.64 (m, 2H), 7.49 (s, 1H), 7.40-7.35 (m, 4H), 7.10 (d, J = 8.0 Hz, 2H), 1.54-1.51 (m, 2H), 1.33-1.27 (m, 2H), 0.90 (t, J = 7.8 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 153.10, 150.50, 141.45, 140.20, 137.67, 136.38, 129.01, 126.44, 125.64, 124.84, 123.84, 119.74, 119.38, 118.81, 116.60, 115.19, 114.51, 34.64, 33.74, 22.18, 14.26. HRMS (ESI) m/z calculated for $[M+H]^+$ 506.2192, found 506.2177.

4.1.1.13. 1-(4-((3-Methoxyacridin-9-yl)amino)phenyl)-3-phenylurea (8m). Yield 79%; yellow solid, mp 233.4-234.8 °C; ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.12 (s, 1H), 9.71 (s, 1H), 9.63 (s, 1H), 8.09 (d, J = 8.0 Hz, 1H), 8.02 (m 1H), 7.93 (dd, J =7.6 Hz, 7.6 Hz, 1H), 7.84 (d, J = 8.0 Hz,1H), 7.71 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.55 (m, 2H), 7.37 (d, J = 8.40 Hz, 2H), 7.31 (dd, J = 8.0 Hz, 7.6 Hz, 2H), 7.25 (d, J = 8.6 Hz, 1H), 7.13 (t, J = 8.0 Hz, 1H), 3.73 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.89, 153.89, 153.12, 140.16, 139.77, 136.06, 134.88, 134.73, 129.27, 128.51, 126.15, 125.93, 124.09, 122.36, 121.52, 119.79, 119.34, 118.61, 115.05, 113.44, 103.92, 56.17. HRMS (ESI) m/z calculated for $[M+H]^+$ 435.1821, found 435.1822.

4.1.1.14. 1-(4-((4-Methoxyacridin-9-yl)amino)phenyl)-3-phenylurea (8n). Yield 75%; yellow solid, mp 227.1-228.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ11.20 (s, 1H), 9.50 (s, 1H), 9.26 (s, 1H), 8.09 (d, J = 8.8 Hz, 1H), 8.03-8.00 (m, 1H), 7.92 (m, J = 7.4 Hz, 1H), 7.72-7.70 (m, 2H), 7.63 (d, J = 8.8 Hz, 2H), 7.48 (d, J = 8.0 Hz, 2H), 7.41 (d, J = 8.0 Hz, 1H), 7.38-7.35 (m, 2H), 7.29 (dd, J = 7.6 Hz, 7.2 Hz, 2H), 6.98 (dd, J =7.6 Hz, 7.6 Hz, 1H), 3.74 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.85, 153.71, 153.11, 140.15, 139.79, 139.64, 134.88, 134.75, 129.25, 128.38, 125.98, 125.90, 124.04, 122.34, 121.61, 119.88, 119.34, 118.60, 115.13, 113.54, 103.92, 56.15. HRMS (ESI) m/z calculated for $[M+H]^+$ 435.1821, found 435.1840.

4.1.1.15.1-(4-((2-Methoxy-6-nitroacridin-9-yl)amino)phenyl)-3-phenylurea (8o). Yield 62%; red solid, mp >300°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.75 (s, 1H), 9.73 (s, 1H), 9.58 (s, 1H), 8.96 (s, 1H), 8.38 (s, 1H), 7.92 (s, 1H), 7.53-7.47 (m, 5H), 7.32 (s, 1H), 7.27 (m, 3H), 7.15 (s, 1H), 7.03 (s, 1H), 6.95 (m, 1H), 4.07 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 165.75, 158.62, 157.48, 152.58, 140.27, 137.35, 129.80, 129.44, 128.88, 128.06, 123.31, 123.01, 121.65, 120.60, 119.22, 115.76, 113.03, 109.88, 46.14.HRMS (ESI) m/z calculated for $[M+H]^+$ 480.1672, found 480.1695.

4.1.1.16. 1-(4-((2-Methoxy-6-nitroacridin-9-yl)amino)phenyl)-3-phenylurea (8p). Yield 77%; yellow solid, mp >300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.68 (s, 1H), 9.69 (d, J = 2.0 Hz, 1H), 9.40 (d, J = 1.6.Hz, 1H), 8.89 (s, 1H), 8.29 (d, J = 9.2 Hz, 1H), 8.10-8.04 (m, 2H), 7.77 (d, J = 7.6 Hz, 2H), 7.64 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 7.6 Hz, 2H), 7.29 (dd, J= 7.6 Hz, 8.0 Hz, 2H), 6.97 (dd, J = 7.2 Hz, 7.2 Hz, 1H), 3.77 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.56, 153.73, 153.04, 149.83, 140.17, 140.07, 138.99, 136.97, 139.38, 129.25, 128.67, 126.05, 122.36, 121.68, 119.33, 118.57, 116.56, 115.87, 115.60, 103.97, 56.30. HRMS (ESI) m/z calculated for $[M+H]^+$ 480.1672, found 480.1692.

4.1.1.17. 1-(4-((6-Chloro-2-methoxyacridin-9-yl)amino)phenyl)-3-phenylurea (8q). Yield 80%;

yellow solid, mp 297.0-298.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H), 9.68 (s, 1H), 9.40 (s, 1H), 8.05 (d, J = 1.6 Hz, 1H), 7.99 (d, J = 9.2 Hz,2H), 7.98 (s, 1H), 7.71(s, 1H), 7.84 (d, J = 9.2 Hz, 2H), 7.64 (t, J = 8.0 Hz, 2H), 7.47(t, J = 8.0 Hz, 1H), 7.38(t, J = 8.0 Hz, 2H),7.29 (t, J = 8.0 Hz, 2H), 7.15(s, 1H), 6.97 (dd, J = 7.6 Hz, 7.2 Hz, 1H), 3.75 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.16, 155.66, 153.88, 140.69, 140.34, 139.30, 133.72, 128.76, 128.58, 128.69, 127.60, 125.98, 124.26, 121.42, 118.93, 118.31, 115.28, 111.80, 104.03, 56.27. HRMS (ESI) m/z calculated for $[M+H]^+$ 469.1431, found 469.1433.

4.1.1.18.1-(4-((2-Methoxyacridin-9-yl)amino)phenyl)-3-(3(trifluoromethyl)phenyl)urea(8r). Yield 78%; yellow solid, mp 233.4-234.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.46 (s, 1H), 9.86 (s, 1H), 9.76 (s, 1H), 8.38 (d, J =8.4 Hz, 1H),8.23 (d, J = 5.6 Hz, 1H), 8.02 (s, 1H), 7.97 (s, 1H), 7.79 (d, J = 6.4 Hz, 1H), 7.62 (d, J = 4.8 Hz, 2H), 7.53 (s, 1H),7.46 (s, 1H), 7.43 (s, 2H), 7.39 (m, 2H), 7.33(s, 1H), 4.16(s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ155.81, 153.15, 148.88, 141.08, 140.35, 139.68, 135.50, 134.82, 132.32, 130.49, 130.27, 126.11, 125.94, 124.48, 124.11, 123.39, 122.21, 120.46, 119.63, 118.67, 117.15, 114.61, 114.09, 113.59, 57.39. HRMS (ESI) m/z calculated for $[M+H]^+$ 503.1695, found 503.1694.

4.2. Molecular docking

The molecular modeling of compound **8a** was performed with Discovery Studio.3.1/CDOCKER protocol (Accelrys Software Inc.) according to the reported process.[23, 36] The dimensional structure of Src (PDB ID: 3G6H) was downloaded from Protein Data Bank (PDB). The following process was used to carry out molecular docking: (1) deleting the water crystallization involved in protein kinase structure; (2) optimizing protein structure and ligands; (3) defining receptor and ligand, finding the candidate binding site; (4) deleting small molecular docking in candidate binding site; (5) docking designed compounds into the candidate binding site on the target protein kinase; (6) molecular modeling based on the above docking data.

4.3. Biological assays

4.3.1. Cell culture

K562 and HepG-2 cells were obtained from Cell Resources Center of Shanghai Institutes for Biological Science, Chinese Academy of Science. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 μgml^{-1} penicillin and 100 μgmL^{-1} streptomycin in humidified air at 37 °C with 5% CO_2.

4.3.2. Cell Viability Assays

4.3.2.1. K562 cells

The synthesized compounds were dissolved in DMSO, and then diluted with culture medium to the final concentrations ranging from 0.01 to 50 μM (the final DMSO concentration was less than 1%) for tumor cells assay. 100 μL of cell solution with the concentration of 7×10^5 cells mL^{-1} was seeded to each well of a 96-well plate and incubated for 12 h at 37 °C in a 5% CO_2 incubator. The test compound solution was added to each well for the treatment of maintained cells in triplicate per concentration, and incubated at 37 °C in a 5% CO_2 incubator for 48 h. After this treatment, 10 μL MTT solution (5 $mgmL^{-1}$) was then added to each well and incubated for 4 h at 37 °C. The formazan precipitate was dissolved in 100 μL DMSO and the absorbance at 495 nm was determined using Multimode Detector DTX880 (Beckman Coulter).

4.3.2.2. HepG-2 cells

The synthesized compounds were dissolved in DMSO, and then diluted with culture medium to the final concentrations ranging from 0.01 to 50 μM (the final DMSO concentration was less than 1%) for tumor cells assay. 100 μL of cell solution with the concentration of 5×10^5 cells mL^{-1} was seeded to each well of a 96-well plate and incubated for 12 h at 37 °C in a 5% CO_2 incubator. The medium was then removed from the 96-well plate. The test compound solution was added to each well for the treatment of maintained cells in triplicate per concentration, and incubated at 37 °C in a 5% CO_2 incubator for 48 h. After this treatment, 10 μL MTT solution (5 $mgmL^{-1}$) was then added to each well and incubated for 4 h at 37 °C. The formazan precipitate was dissolved in 100 μL DMSO

and the absorbance at 495 nm was determined using Multimode Detector DTX880 (Beckman Coulter).

4.3.3. Kinase Inhibition Assays

In vitro kinase assays were carried out by Medicilon Co., Ltd in Shanghai, China. Kinases inhibitory activities of synthetic compounds against Src and MEK. The general procedures were as follows: mix enzyme, substrate, ATP and compounds in a buffer solution (pH 7.0) of 50 mM HEPES/NaOH, 0.02% NaN_3, 0.01% BSA, 0.1m Mortho-vanadate, 5 mM $MgCl_2$, 1 mM DTT in an OptiPlate-384. The assay plate was incubated at room temperature for 15 min and compounds in 10 μM dissolved in 2.5% DMSO. Then the mixture was added by Streptavidin-XL665 and TK antibody europium cryptate (1:100) solution 10 μL (50 mM HEPES/NaOH pH 7.0, 0.1% BSA, 0.8 M KF, 20 mM EDTA). The Instrument (Perkinelmer) to detect the signal at room temperature. The luminescence was read at envision. The signal was correlated with the amount of ATP remaining in the reaction and was inversely correlated with the kinase activity.

4.3.4. Cell Apoptosis Assays

A total of 1×10^5cells mL^{-1} K562 cells were plated in a six-well plate and treated with **8m** for 48 h at 37 °C. After incubation, the cells were harvested and washed with ice-cold PBS. The cell cycle progression was analyzed using a cell cycle and apoptosis analysis kit (Beyotime), and the apoptosis ratio was performed with an annexin V-FITC Apoptosis Detection Kit (keygentec).

4.3.5. Western Blot Analysis

After treatment with a series of concentrations of **8m** for 48 h at 37 °C, K562 cells were harvested, a centrifuge separates the cells from substratum at the speed of 800 rap for 5min. Removed supernate, lysed (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 30 min. Whole-cell protein lysates were prepared and centrifuged for 10 min at 12000 g and 4 °C to remove any insoluble material. The total proteins were determined using the Bradford method, and an equivalent quantity of protein was combined with an SDS−PAGE loading buffer in boiled water for

5 min. Cell lysates were separated by SDS−PAGE and electro-transferred onto PVDF membranes (amcBiobind NT-200). The PVDF membranes were incubated with each antibody and detected according to the immunoblot analysis principle. The antibodies were purchased from Cell Signaling Technology, and the dilutions of the antibodies were according to the instruction from Cell Signaling Technology.

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Supplementary data

Supplementary data (Molecular modeling of compound **8m** with Src and MEK proteins, Synthesis of compounds **4a-4g** and **7a-7l,** the UV-vis absorption spectra of compound **8m** binding with ctDNA**,** ^{1}H NMR and ^{13}C NMR spectra and high resolution mass spectrometry of **8a-8r**) associated with this article can be found, in the online version.

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Legends

Figure 1.the structure of compound **OA**.

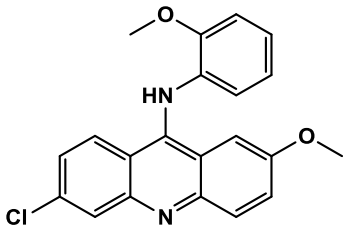
Figure 2. (a) the structure of compound **8a**; (b) Molecular modeling of compound **8a** binding to Src protein (PDB ID: 2OIQ); (c) Molecular modeling of compound **8a** binding to MEK protein (PDB ID: 4LMN).

Figure 3. 8m affected the expression of ERK and AKT in cell cultures. K562 cells were treated with compound **8m** for 48 h. Cells were lysed, and the proteins were analyzed by Western blot analysis.

Figure 4. 8m induced apoptosis of K562 cells. K562 cells were harvested after treatment with various concentrations of compound **8m** for 48 h. Cells were stained with an annexin V-FITC apoptosis detection kit.

Figure 5. 8m affected the expression of C-Caspase-3, C-caspase-7, C-PARP in cell cultures. K562 cells were treated with compound **8m** for 48 h. Cells were lysed, and the proteins were analyzed by Western blot analysis.

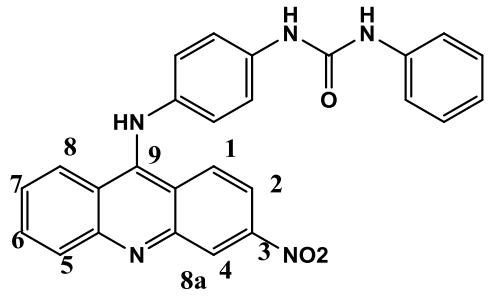
Scheme 1. Reagents and conditions: a. (i) Cu, K_2CO_3, DMF, 130 ℃, overnight; (ii) $POCl_3$, reflux, 3 h; b. (i) different substituent of aniline, Et_3N, THF, reflux, 3-4 h; (ii) Fe, NH_4Cl, EtOH, 2 h; c. (i) HCl, THF, r.t., 2 days.



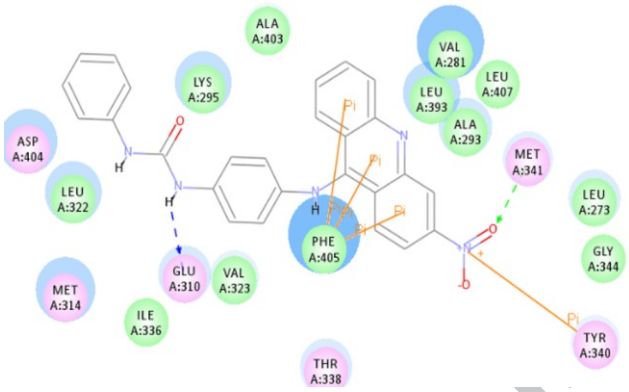
compound OA

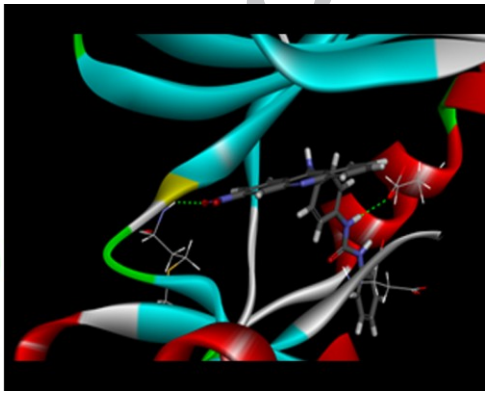
Figure 1

(a)

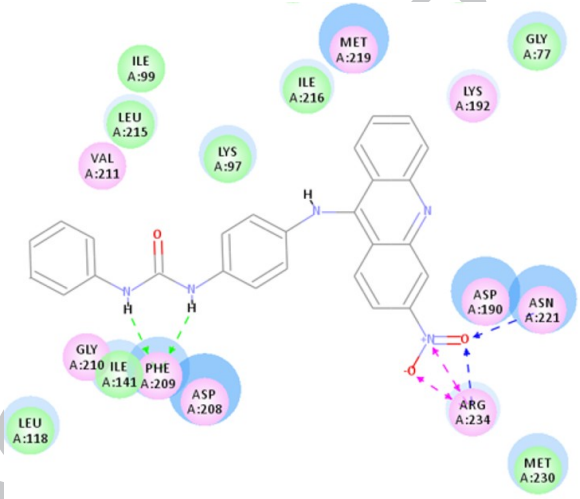


(b)





(c)



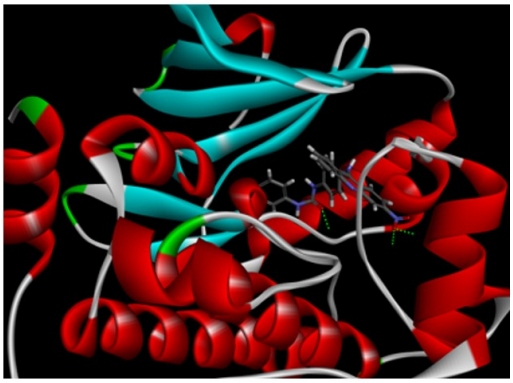


Figure 2

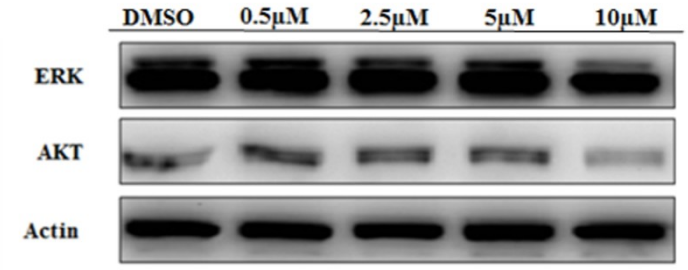


Figure 3

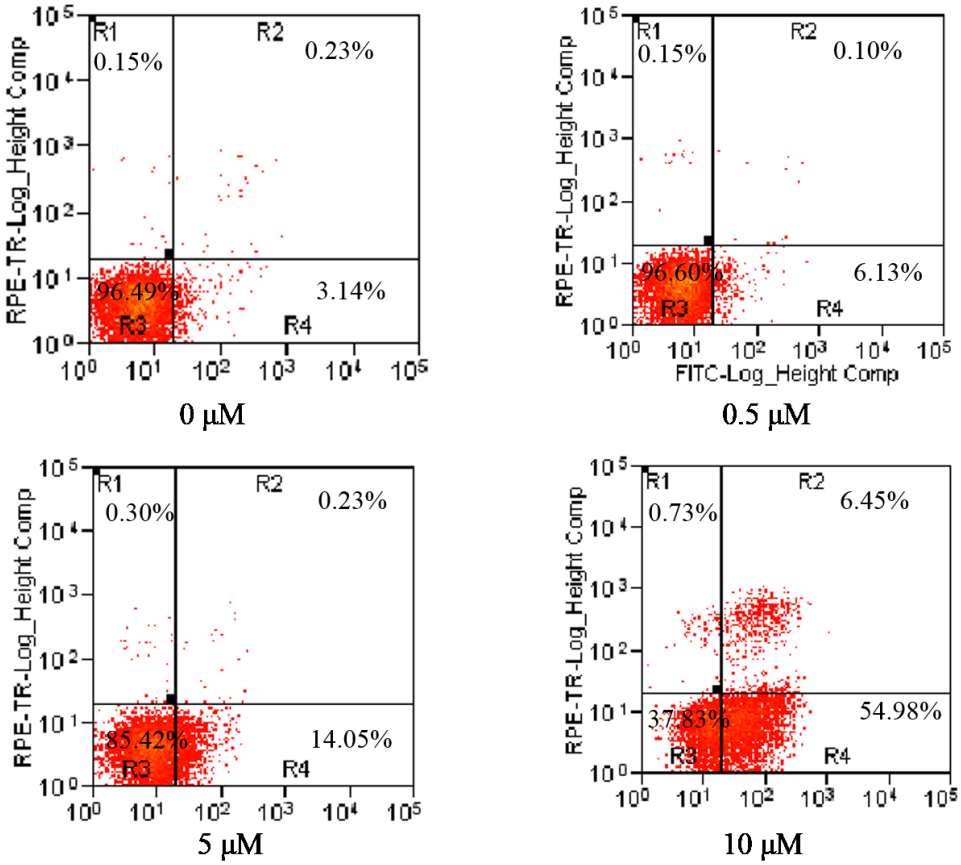


Figure 4

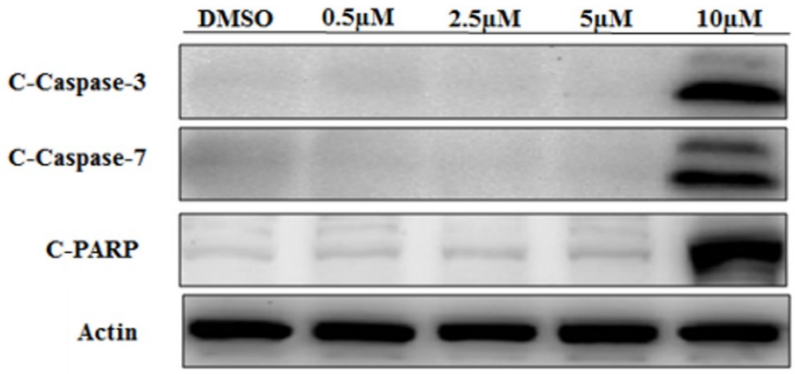
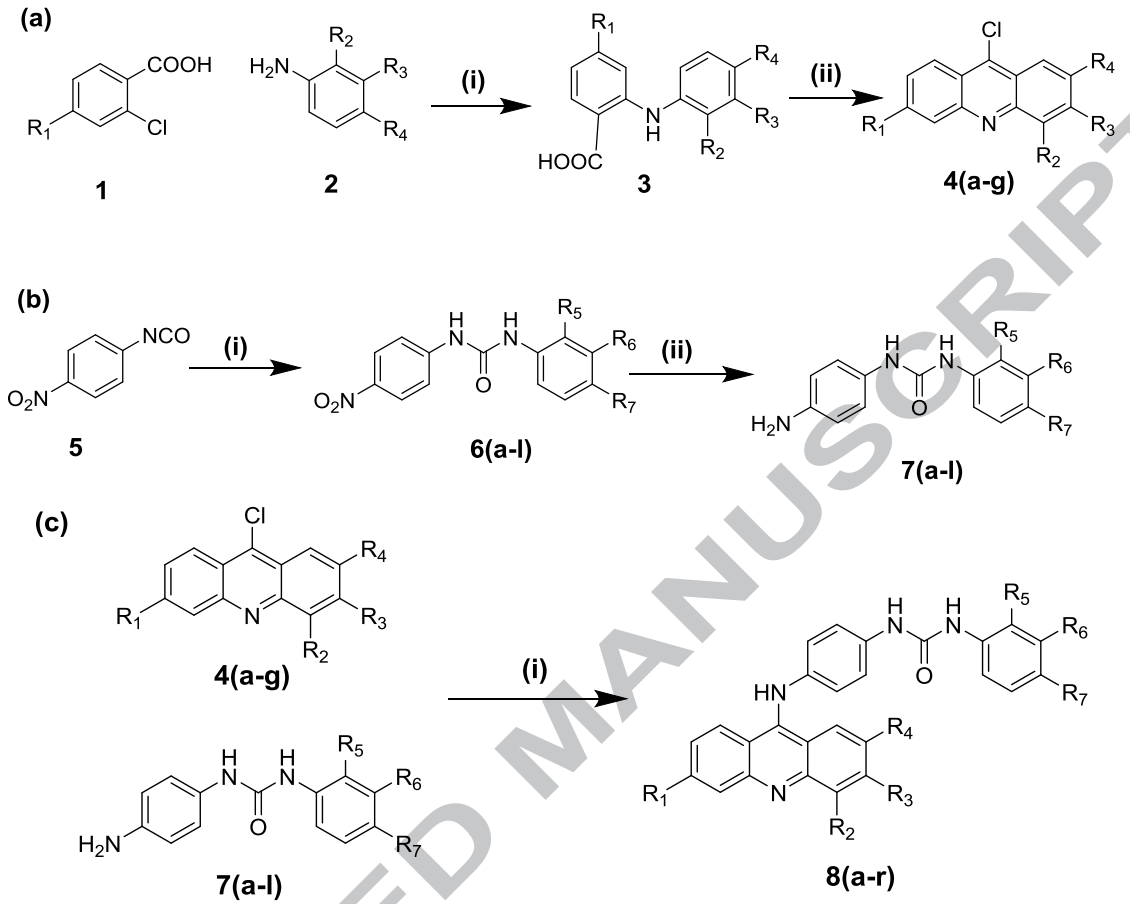


Figure 5



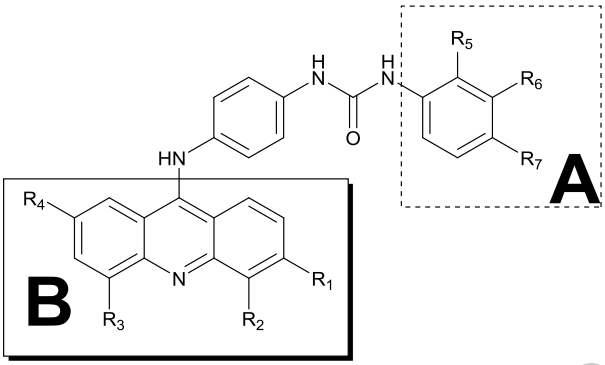
Scheme 1

Table 1. Molecular docking data of compound **8a**.

	Src		MEK	
	Energy[a]（kcal/mol）			
	-CDocker	-Interaction	-CDocker	-Interaction
8a	17.86	60.62	24.44	62.49
OA	15.01	50.89	13.25	47.59

Energy[a]: Evaluation of the molecular docking in Discovery Studio.3.1/CDOCKER Model was performed according to the scores of two scoring functions, including CDOCKER Energy and CDOCKER Interaction Energy. The greater values indicated that molecules have better protein binding affinity.

Table 2. Anti-proliferation activities against K562 and HepG-2 cells of compounds **8a–8r**.

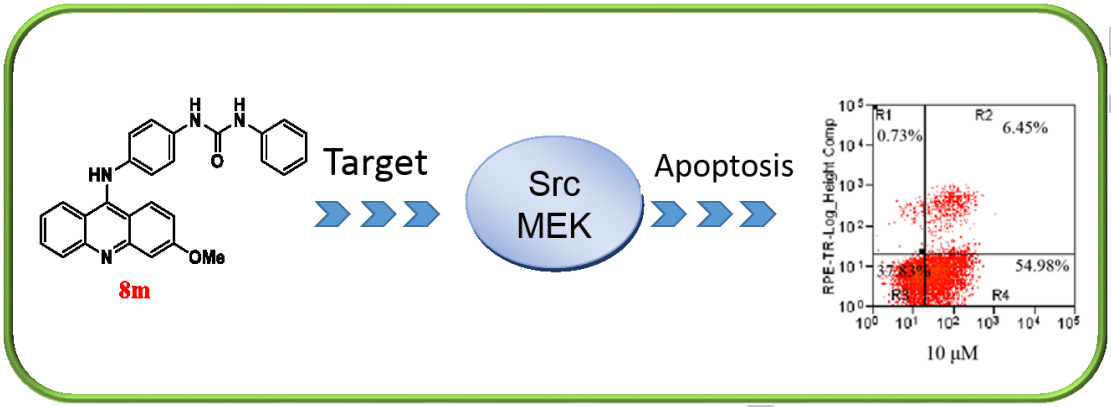


Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	K562 IC_{50}(μM)	HepG-2 IC_{50}(μM)
8a	NO_2	H	H	H	H	H	H	3.30±0.89	19.79±2.22
8b	NO_2	H	H	H	OMe	H	H	4.51±0.56	19.35±3.50
8c	NO_2	H	H	H	H	OMe	H	4.55±1.32	22.32
8d	NO_2	H	H	H	H	H	OMe	7.68±0.12	>25
8e	NO_2	H	H	H	H	F	H	11.08±2.57	>25
8f	NO_2	H	H	H	H	Cl	H	13.89±1.38	>25
8g	NO_2	H	H	H	H	CF_3	H	1.20±0.14	>25
8h	NO_2	H	H	H	H	CH_3	H	11.60±1.63	>25
8i	NO_2	H	H	H	H	C_2H_5	H	8.92±0.26	22.69±1.29
8j	NO_2	H	H	H	H	H	C_2H_5	1.86±0.24	14.77±1.46
8k	NO_2	H	H	H	H	H	C_3H_7	1.56±0.01	14.37±1.17
8l	NO_2	H	H	H	H	H	C_4H_9	1.23±0.01	14.00±1.76

8m	OMe	H	H	H	H	H	H	4.08±0.14	9.41±1.09
8n	H	OMe	H	H	H	H	H	0.49±0.01	6.12±0.52
8o	NO_2	H	OMe	H	H	H	H	2.58±0.19	17.63±1.90
8p	NO_2	H	H	OMe	H	H	H	3.79±0.17	>25
8q	Cl	H	H	OMe	H	H	H	3.42±0.15	9.56±2.07
8r	H	OMe	H	H	H	CF_3	H	1.93±0.01	13.12±0.11
OA								5.8±0.05	>50
Imatinib								0.53±0.01	>25

Graphical abstract

Design, Synthesis and Evaluation of Acridine Derivatives as Multi-Target Src and MEK Kinase Inhibitors for Anti-tumor Treatment



A series of novel multi-target inhibitors of Src and MEK bearing acridine and phenyl-urea scaffolds were designed and synthesized based on molecular docking method.