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Novel synthetic acridine derivatives as potent DNA-binding and apoptosis-inducing antitumor agents

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ABSTRACT

Acridine derivatives have been explored as DNA-binding anticancer agents. Some derivatives show undesired pharmacokinetic properties and new derivatives need to be explored. In this work, a series of novel acridine analogues were synthesized by modifying previously unexplored linkers between the acridine and benzene groups and their antiproliferative activity and the DNA-binding ability were evaluated. Among these derivatives, compound **5c** demonstrated DNA-binding capability and topoisomerase I inhibitory activity. In K562 cell lines, **5c** induced apoptosis through mitochondria-dependent intrinsic pathways. These data suggested that compound **5c** and other acridine derivatives with modified linkers between the acridine and benzene groups might be potent DNA-binding agents.

1. Introduction

Cancer has become the main cause of mortality and there is an urgent need for more effective anticancer drugs.[1,2] Extensive efforts have been directed at the development of new drugs with promising anticancer properties. DNA targeted drugs are a class of clinically highly successful anticancer agents that produce anticancer effects by causing DNA damage and subsequent cell apoptosis,[3,4] having significantly increased the survival rate of cancer patients.[5] Nonetheless, a main drawback that clinically-used DNA targeted agents frequently encounter is drug resistance, which has led to the efforts in discovering new agents that can bind to DNA and DNA regulatory enzymes, particularly the agents effective against drug resistant cancers.[6–12]

Previous reports have demonstrated that a planar chromophore, such as a tri- or tetracyclic ring, is a fundamental structural element for DNA intercalation, one of the major interaction modes by known DNA binding agents.[13] Acridine is one of the extensively explored DNA intercalating scaffolds for developing anticancer, antiviral, antimalarial, and antibacterial agents, and for biological fluorescent probes.[14–17] In particular, the 9-anilinoacridine derivative *m*-amsacrine (***m*-AMSA**, Fig. 1) has attracted much attention for its DNA-binding capability.[18] Because of the short half life of *m*-AMSA in the presence of fresh mouse blood at 37 °C, various analogues, such as 3-(9-acridinylamino)-5-hydroxymethylaniline (**AHMA**, Fig. 1), have been designed and tested for anticancer activities.[19] Thus far, most of *m*-AMSA derivatives are 9-anilinoacridines.[20–22] Little attention has been paid to the derivatives with modified linkers between the acridine and benzene groups.[23]

As part of our continuous efforts for developing anticancer compounds,[24–33] in this work, we synthesized new *m*-AMSA derivatives (Table 1; **5a**–**5f**, **6b**, **7b**) with modified linkers between the acridine and benzene groups for the discovery of potent DNA-binding agent and evaluated the structure–activity relationship of these derivatives. This study is based on our earlier work in the discovery

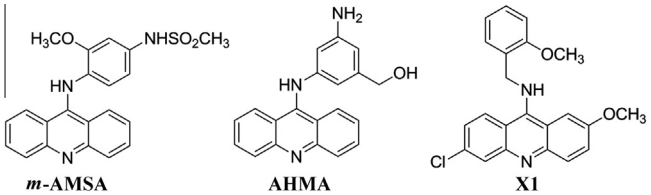

Figure 1. Chemical structures of *m*-AMSA, AHMA, and compound **X1**.

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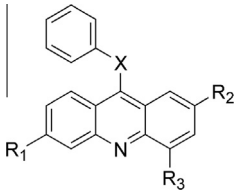
Table 1
Antiproliferative activity in K562 and HepG-2 cells



Compound	R_1	R_2	R_3	X	K562 IC_{50} (μM)	HepG-2 IC_{50} (μM)
5a-1	Cl	OCH_3	H	CH_2–NH	0.65	1.40
5a-2	Cl	OCH_3	H	$(CH_2)_2$–NH	0.65	2.19
5a-3	Cl	OCH_3	H	$(CH_2)_3$–NH	2.20	3.26
6b	H	H	H	CH_2–O	>50	>50
7b	H	H	H	CO–NH	>50	>50
5b	H	H	H	CH_2–NH	5.24	6.07
5c	H	OCH_3	H	CH_2–NH	0.54	0.89
5d	Cl	H	H	CH_2–NH	9.24	11.64
5e	Cl	OCH_3	CH_3	CH_2–NH	3.09	6.05
5f	H	H	CH_3	CH_2–NH	4.86	3.64
Imatinib					0.47	ND[a]
Colchicin					ND[a]	1.80

[a] ND: not detected.

of 9-(2-methoxybenzyl) acridine derivative compound **X1** (Fig. 1) as an antiproliferative agent[24] and on the synthesis of 9-anilinoazaacridines derivatives with chloro and methoxy groups substituted at C-2 and C-6 positions of acridine ring, respectively.[29] The topoisomerase I (topo I) inhibitory activity and cytotoxicity against K562 and HepG-2 cells of new compound **5c** represents the success of this exploration in the linker modifications of acridine derivatives.

2. Results and discussion

2.1. Chemistry

Synthesis of the acridine derivatives **5** were accomplished as described in Scheme 1(a). Compounds **3** (**3a**–**3f**) were obtained from an Ullmann reaction of benzoic acids **1** with the anilines **2** in DMF using Cu as the catalyst under basic condition. Compounds **3** (**3a**–**3f**) were stirred in $POCl_3$ giving the 9-chloroacridine derivatives **4** (**4a**–**4f**). The compounds **5** (**5a**–**5f**) were obtained by the reaction of substituted benzylamines and compounds **4** using KI and K_2CO_3 in absolute ethanol under reflux conditions.

In Scheme 1(b) and (c), the synthesis of compounds **6b**, **7b** by the same synthetic method failed due to the low nucleophilic activity of benzyl alcohol and benzamide. Therefore a stronger base such as NaH was used. Compound **6b** was achieved by compounds **4b** with benzyl alcohol in the presence of NaH and catalytic amounts of potassium iodide in THF under reflux conditions. The compound **7b** was obtained by the reaction of **4b** and benzamide according to the routes described in the references.[34]

2.2. In vitro cytotoxicity

In vitro cytotoxicity of the 10 acridine derivatives was evaluated against K562 leukemia cells and hepatoma HepG-2 cells by MTT assay using imatinib and colchicin as the positive controls. As shown in Table 1, most of the compounds had low micromolar IC_{50} values, among which compound **5c** showed the best activity against both K562 and HepG-2 cells with IC_{50} values at 0.54 and 0.89 μM, respectively.

Data in Table 1 indicate that the modification of the linker between acridine ring and benzene ring and the substituents of the acridine significantly influenced the cytotoxicity. First, the length of the alkyl chain between the 9-anilinoacridine and benzene groups has modest influence on the cytotoxicity as indicated by the measured activities of compounds **5a-1**, **5a-2** and **5a-3**. The compound **5a-1** with the shortest chain length showed better cytotoxicity than those of **5a-2**, **5a-3**. It seems that increase in the length of the alkyl chain leads to modest reduction of cytotoxicity. Second, a substitution of alternative linkers, that is, compounds **6b** comprising an alkoxy linker and **7b** containing an acylamino linker led to decrease in antiproliferative activity. Finally, we investigated the modifications in the acridine ring. The cytotoxic activity of compound **5c** with a methoxy group substituted at C-2 position of acridine ring was almost 10 times higher in potency than that of **5b** with no substituents of the acridine ring. The compounds **5d** and **5a-1** showed the similar or slightly reduced activity, comparing with **5b** and **5c**, respectively. These suggest that a chloro group substituted at C-6 position of acridine ring has no obvious effect on the activity of this class of derivatives, but a methoxy group substituted at C-2 position of acridine ring significantly altered their activity. On the other hand, a methyl group on the C-4 position of acridine ring appeared to be unimportant for the activity by the fact that **5f** was more active than **5b** while **5e** was less active than **5a-1**.

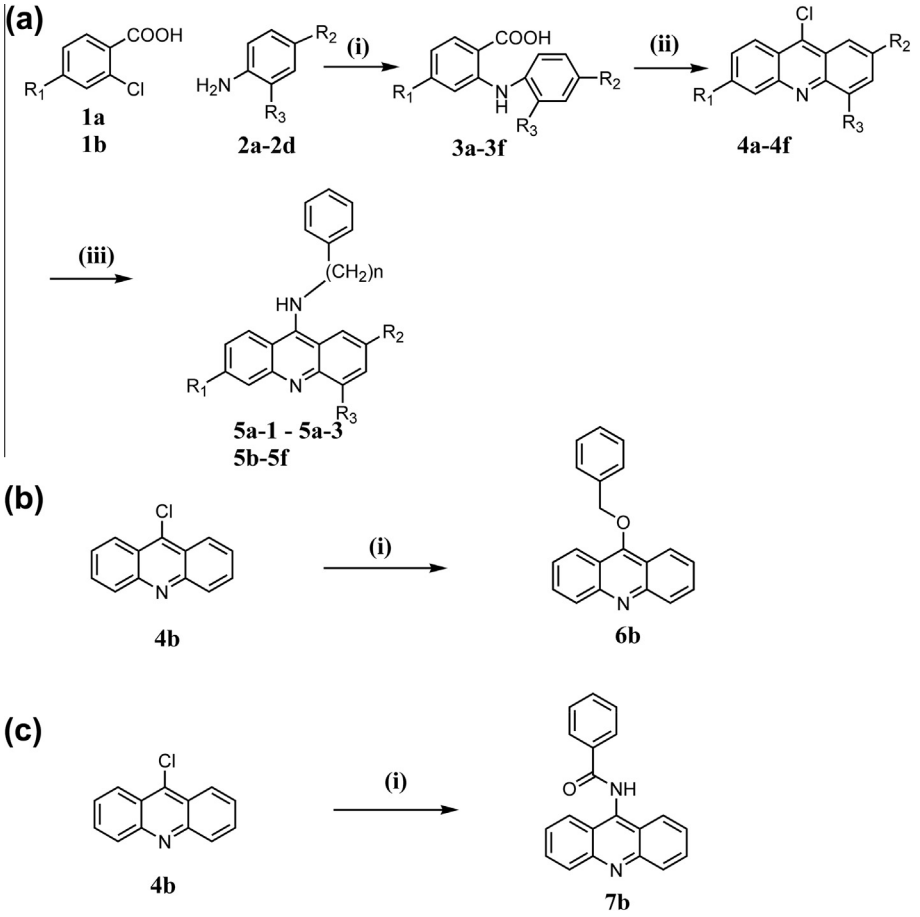
2.3. Fluorescence spectra

Fluorescence spectroscopy has been widely applied for investigating the interaction between drugs and DNA.[6] All compounds were gradually mixed with increasing equivalent of deoxyribonucleic acid from calf thymus (ct DNA) in Tris–HCl at pH 7.0. The typical spectra were shown in Figure 2(a) and the other spectra can be seen in Figure 1s (Supporting information). The fluorescence of acridine derivatives (50 μM) were gradually quenched with the increasing amounts of ct DNA, suggesting the interaction between acridines and ct DNA.

We used the classical Stern–Volmer Eq. (1)[35] to calculate the quenching constant K_q for the binding of the compound with DNA, which was usually in the range of 10^4–10^6 M^{-1}.

$$F_0/F = 1 + K_q[Q] \quad (1)$$

The quenching constant K_q for compound **5c** was shown in Figure 2(b). The K_q of all the synthesized compounds were shown in Table 2. From the results of fluorescence spectroscopy, most of



Scheme 1. Reagents and conditions: (a) (i) K_2CO_3, Cu, DMF, 130 °C; (ii) $POCl_3$, 140 °C; (iii) various benzylamines, K_2CO_3, KI, ethanol, reflux. (b) (i) Benzylalcohol, NaH, KI, THF, reflux. (c) (i) Benzamide, NaH, DMF, reflux.

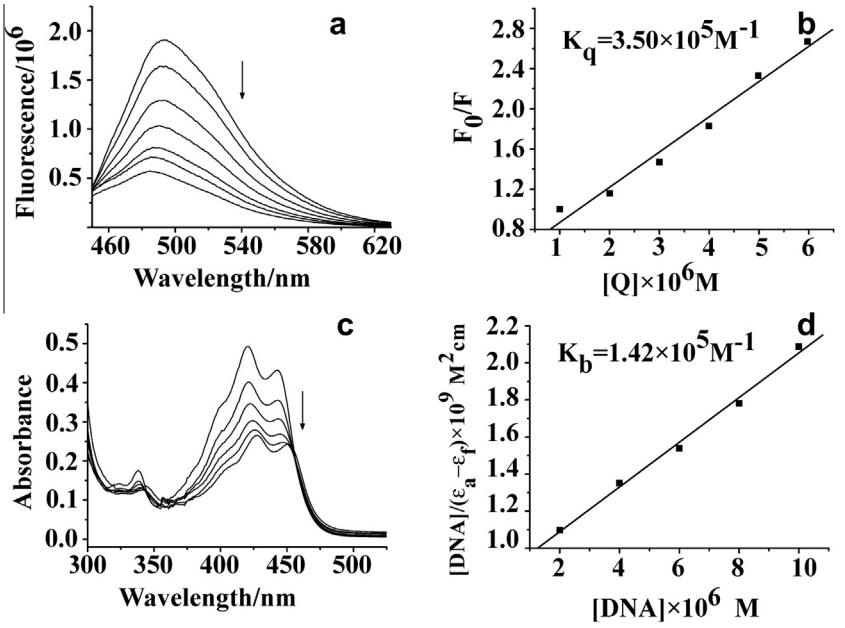


Figure 2. (a) Spectrofluorimetric titration of compound **5c** (50 μM) in 10 mM Tris–HCl buffer containing 10 mM NaCl (pH 7.0) by increasing the concentrations of ct DNA ([DNA] = 0, 1, 2, 3, 4, 5, 6 μM). The arrow indicates the fluorescence emission changes upon increasing DNA concentrations. (b) Stern–Volmer quenching plots of the fluorescence titration, $K_q = 3.50 \times 10^5\ M^{-1}$. (c) UV–vis absorption spectra of compound **5c** (50 μM) in the presence of increasing amounts of ct DNA; [DNA] = 0, 2, 4, 6, 8, 10 μM. DNA titration of the compound was performed in 10 mM Tris–HCl buffer containing 10 mM NaCl at pH 7.0. The arrow indicates the absorbance changes upon increasing DNA concentrations. (d) The plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ as a function of DNA concentrations as determined from the absorption data.

the compounds can interact with DNA. The compound **5a-1** with the shortest chain length had better binding capacity than those of **5a-2**, **5a-3**. Compounds **6b** and **7b** with little antiproliferative activity had low DNA-binding capacity. Compound **5c** displayed the highest DNA binding activity. The results were generally consistent with the results of cell growth inhibition assay.

2.4. UV–visible spectral absorbance

In addition to the electronic fluorescence spectroscopy, the DNA binding capacity was also detected by the absorption spectroscopy. Due to the strong stacking interaction between an aromatic chromophore and the base pairs of DNA, compound binding to DNA can result in hypochromism and red-shifting in the absorption of the compound. The absorption spectra of **5c** in the absence and presence of ct DNA were presented in Figure 2(c). With the increase of the concentration of ct DNA, the intensity of the spectrum of **5c** was decreased obviously. In addition, we also detected an obvious bathochromic shift of the chromophore in the absorbance, which suggested that the compound **5c** interact with DNA.

We used formula (2) to calculate the constant K_b for the binding of the compound with DNA,[36] where [DNA] represents the DNA concentrations in base pair, while ε_a, ε_f and ε_b are the extinction coefficient of the compound absorption band at a given DNA concentration, the complex free in solution and the complex when fully bound to DNA, respectively.[36] When the equilibrium is reached to an optimum level and the intensity of the spectrum is not obviously decreased when more DNA were added, which seems to indicate that the compound is fully bound to DNA. The K_b was calculated by the gradient ratio to the intercept. The binding constant K_b of compound **5c** was $1.42 \times 10^5\ M^{-1}$, which is generally consistent with the data of fluorescence spectroscopy.

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (2)$$

2.5. CD spectroscopy

Circular dichroic (CD) spectral technique was used to search for the conformational changes of ct DNA on addition of compound **5c** (Fig. 3). The CD spectrum of ct DNA produced a classical diagram of right-handed B-form DNA with positive and negative bands at 275 and 248 nm due to base-stacking and helicity, respectively.[31] The increased intensity of negative bands with slight red shifts were observed with increasing amount of compound **5c**. The positive band at 275 nm was also increased with no significant red shift when the concentration of **5c** was increased. The enhancement of the two bands indicated that **5c** interacts with DNA. Slight or no red shifts suggested the binding of **5c** did not lead to any significant change in conformation of ct-DNA.[37,38]

2.6. DNA topoisomerase I inhibition assay

Because of the capabilities of acridine derivatives to interact with ct DNA, it is of interest to evaluate whether they can also inhibit some of the nuclear enzymes involved in DNA processing, such as topo I. Figure 4 shows the relative affinity of all compounds on the relaxation of plasmid pBR322 DNA mediated by topo I. Most

Table 2
The quenching constants K_q for the binding of all compounds with DNA

Compound	**5a-1**	**5a-2**	**5a-3**	**6b**	**7b**
K_q	1.97×10^5	1.19×10^5	1.05×10^5	$<1.00 \times 10^4$	$<1.00 \times 10^4$
Compound	**5b**	**5c**	**5d**	**5e**	**5f**
K_q	3.71×10^4	3.50×10^5	1.33×10^4	1.11×10^5	6.22×10^4

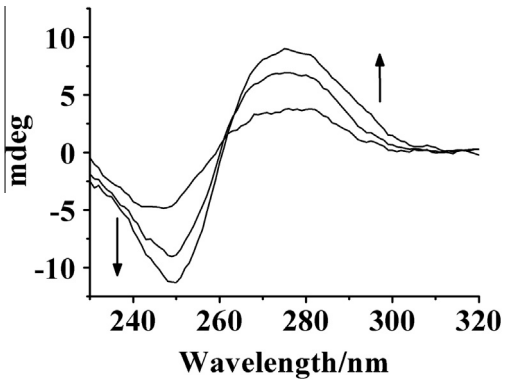


Figure 3. Circular dichroism spectra of ct DNA (40 μM) by increasing the concentrations of **5c** (0, 40, 80 μM).The arrow indicates the CD spectra changes upon increasing the concentrations of compound **5c**.

of the compounds with DNA-binding capacity displayed certain topo I inhibitory activity at 100 μM. In addition, compounds **5b**, **5c** and **5f** which displayed good cytotoxicity against K562 and HepG-2 cells, showed good topo I inhibitory activity at 25 μM. These data suggest that these compounds may have antiproliferative activity by interacting with DNA and inhibiting topo I, and some of their derivatives may thus be developed into potent topo I inhibitors.

2.7. Apoptosis induced by compound 5c

Our spectroscopic studies consistently suggested that compound **5c** may bind with DNA and inhibit topo I activity, which was expected to subsequently lead to apoptosis in cancer cells.[39] To test this hypothesis, an Annexin-V/PI binding assay was conducted in K562 cells. As shown in Figure 5, the upper and lower right-hand quadrants represented the late stage apoptotic cells (Annexin-V and PI positive) and the early stage apoptotic (Annexin-V positive but PI negative). K562 cells were treated with **5c** at the concentrations of 0, 0.5, 1.0 and 2.5 μM for 48 h. As the concentration of **5c** increased, the early and the late stage apoptotic cells increased from 4.32% to 73.15%, indicating that **5c** induces apoptosis of K562 cells in a dose-dependent manner.

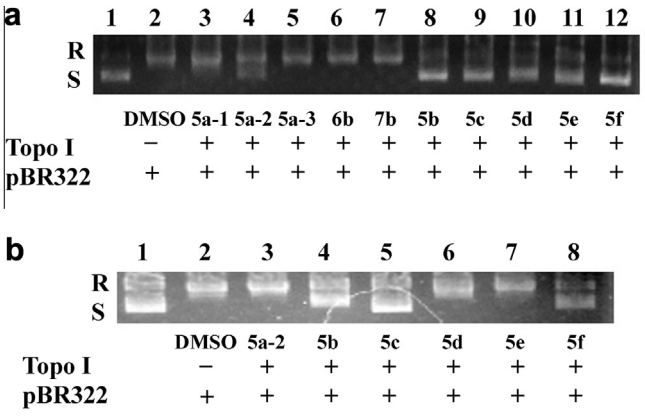


Figure 4. Effect of the compounds on the relaxation of plasmid DNA by human topoisomerase I. (a) Lane 1, DNA pBR322; Lane 2, topo I + DNA pBR322 + DMSO; Lanes 3–12, DNA pBR322 relaxation by topo I and **5a-1**, **5a-2**, **5a-3**, **6b**, **7b**, **5b**, **5c**, **5d**, **5e** and **5f** at concentrations of 100 μM, respectively. (b) Lane 1, DNA pBR322; Lane 2, topo I + DNA pBR322 + DMSO; Lanes 3–8, DNA pBR322 relaxation by topo I and **5a-2**, **5b**, **5c**, **5d**, **5e** and **5f** at concentrations of 25 μM, respectively.

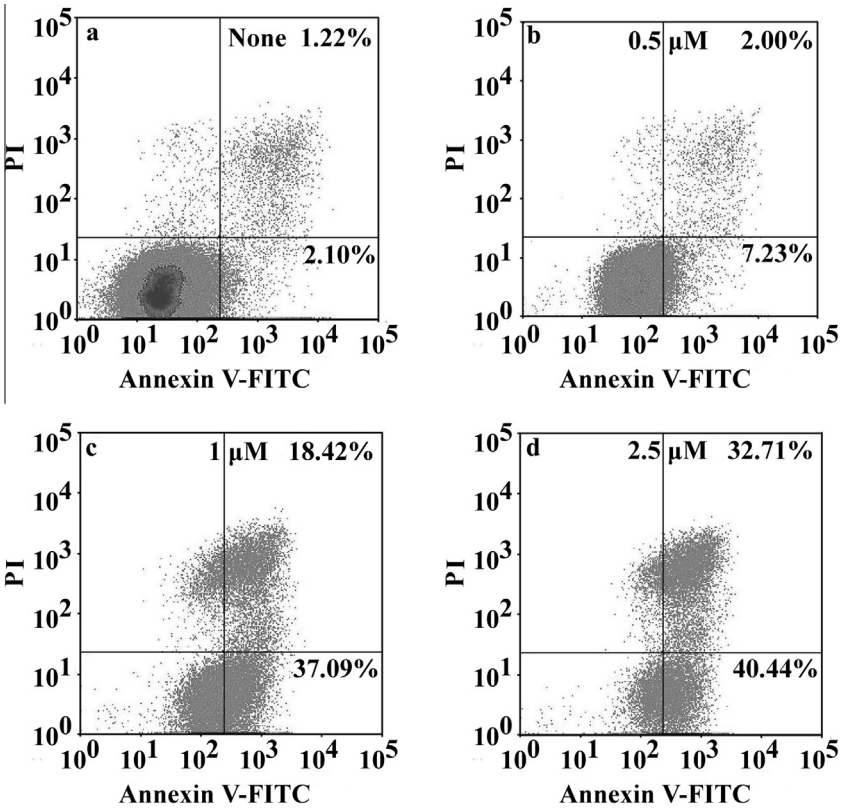


Figure 5. Flow cytometric analysis of phosphoatidylserine externalization (Annexin-V binding) and cell membrane integrity (PI staining). K562 cells were treated with compound **5c** at 0, 0.5, 1, 2.5 μM, respectively.

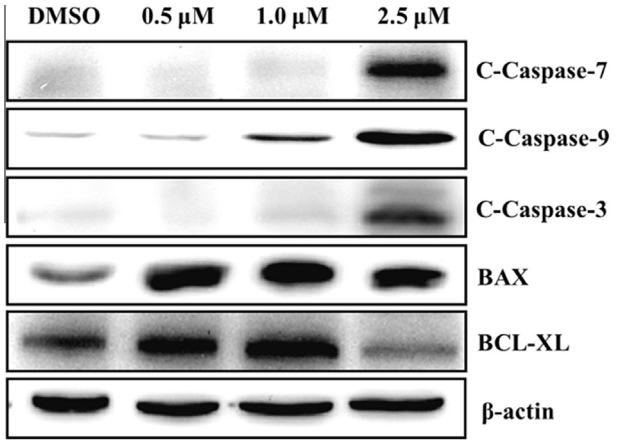


Figure 6. K562 cells were treated with different concentrations of compound **5c**, and the levels of C-caspase-7, C-caspase-9, C-caspase-3, BAX, BCL-XL were determined by Western blot analysis.

We further investigated apoptotic proteins to further confirm that compound **5c** induces apoptosis and to dissect which parts of the apoptotic pathway are affected by the compound. We first studied caspases, a family of cystein proteases, can cleave essential cellular substrates after aspartic residues and are critical initiators and effectors of apoptosis. Caspase-3 and caspase-7 are effectors activated by upstream initiators of apoptosis and play an irreplaceable role in cell apoptosis and caspase-9 is involved in the mitochondrial pathway. As shown in Figure 6, compound **5c** at 2.5 μM for 48 h induced significant cleavages of caspase-3 and caspase-7. Compound **5c** also induced the activation of caspase-9 in a dose-dependent manner, indicating a mitochondrial-dependent apoptosis. Based on this finding, we went on to study Bcl-2 family proteins. The Bcl-2 family proteins play critical roles in controlling the mitochondrial pathway. Either increase of pro-apoptotic protein Bax or decrease of anti-apoptotic protein BCL-XL promotes cell apoptosis. As shown in Figure 6, compound **5c** at 2.5 μM effectively increased Bax and decreased BCL-XL protein, illustrating that compound **5c** induced apoptosis may proceed through a caspase-dependent intrinsic mitochondria pathway.

3. Conclusion

A series of novel acridine analogues were synthesized by modifying the previously unexplored linkers between the acridine and the benzene group, and most of them showed promising cytotoxicity in K562 and HepG-2 cells. In particular, compound **5c** displayed highest cytotoxicity against both K562 and HepG-2 cells. DNA-binding and DNA topo I inhibition assay demonstrated that **5c** interacted with ct DNA and inhibited topoisomerase I activity, and induced apoptosis in K562 cells via the caspase-dependent intrinsic mitochondria pathway. Our studies suggested that compound **5c** is a highly promising lead compound for further optimization into potentially potent DNA binding and apoptotic inducing agents. Its derivatives may also be developed into DNA topo I inhibitors. Further modifications of **5c** are underway.

4. Experimental section

4.1. Synthesis and characterization

See Supporting information for synthetic methods and the preparation of compounds **3a**–**3f** and **4a**–**4f**.

4.1.1. General procedure for compounds 5a-1–5a-3, 5b–5f

Various amines (2.00 mmol) were dissolved in absolute alcohol (15 mL) and then potassium carbonate (2.00 mmol) was added. The mixture was stirred for 45 min at room temperature. The compound **4**(**a**–**f**) (1.00 mmol) and potassium iodide (0.25 mmol) were added. The mixture was stirred and refluxed overnight. Then the mixture was poured into water (50 mL), extracted with ethyl acetate to give the crude product. The crude product was purified by column chromatography using petroleum ether and ethyl acetate (5:1 v/v).

4.1.1.1. 6-Chloro-2-methoxy-*N*-benzylacridin-9-amine (5a-1). Yield 82%; mp 158–160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 1.7 Hz, 1H), 8.01 (d, J = 9.4 Hz, 1H), 7.96 (d, J = 9.3 Hz, 1H), 7.46–7.27 (m, 7H), 7.13 (d, J = 2.5 Hz, 1H), 4.92 (s, 1H), 4.84 (s, 2H), 3.77 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.27, 149.44, 148.36, 147.13, 139.39, 134.84, 131.68, 129.09, 128.51, 128.01, 127.48, 125.08, 124.84, 123.76, 118.42, 116.43, 99.23, 55.39, 54.79; HR-MS(ESI): calcd for $C_{21}H_{17}ClN_2O$ $[M+H]^+$ 349.1107; found: 349.1096.

4.1.1.2. 6-Chloro-2-methoxy-*N*-phenethylacridin-9-amine (5a-2). Yield 67%; mp 122–124 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 1.9 Hz, 1H), 7.98 (d, J = 9.4 Hz, 1H), 7.86 (d, J = 9.3 Hz, 1H), 7.44–7.33 (m, 3H), 7.29 (t, J = 6.7 Hz, 3H), 7.25 (d, J = 2.0 Hz, 1H), 4.64 (s, 1H), 3.96 (t, J = 6.4 Hz, 2H), 3.82 (s, 3H), 3.03 (t, J = 6.6 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.26, 149.27, 148.42, 146.84, 138.12, 134.74, 131.64, 129.01, 128.98, 128.38, 127.07, 124.73, 124.70, 124.17, 118.53, 116.46, 98.53, 55.46, 51.10, 37.24; HR-MS(ESI): calcd for $C_{22}H_{19}ClN_2O$ $[M+H]^+$ 363.1264; found: 363.1263.

4.1.1.3. 6-Chloro-2-methoxy-*N*-phenethylacridin-9-amine (5a-3). Yield 78%; mp 79–81 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (s, 1H), 7.96 (d, J = 9.3 Hz, 1H), 7.81 (d, J = 9.2 Hz, 1H), 7.37 (d, J = 8.8 Hz, 1H), 7.19 (ddd, J = 38.0, 13.5, 6.6 Hz, 7H), 4.67 (s, 1H), 3.87 (s, 3H), 3.66 (t, J = 6.8 Hz, 2H), 2.71 (t, J = 7.2 Hz, 2H), 2.05 (dd, J = 14.0, 6.9 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.04, 149.47, 148.34, 146.81, 140.91, 134.72, 131.51, 128.65, 128.35, 128.27, 126.30, 124.52, 123.88, 117.99, 115.92, 99.25, 55.52, 50.03, 33.29, 33.18; HR-MS(ESI): calcd for $C_{23}H_{21}ClN_2O$ $[M+H]^+$ 377.1420; found: 377.1410.

4.1.1.4. *N*-benzylacridin-9-amine (5b). Yield 76%; mp 137–140 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, J = 8.5 Hz, 2H), 8.02 (d, J = 8.7 Hz, 2H), 7.67–7.57 (m, 2H), 7.38–7.22 (m, 7H), 5.25 (s, 1H), 4.89 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 151.14, 149.22, 139.31, 129.87, 129.25, 128.98, 127.89, 127.58, 123.23, 122.84, 117.05, 55.04; HR-MS(ESI): calcd for $C_{20}H_{16}N_2$ $[M+H]^+$ 285.1391; found: 285.1400.

4.1.1.5. 2-Methoxy-*N*-benzylacridin-9-amine (5c). Yield 31%; mp 179–180 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (d, J = 8.7 Hz, 1H), 7.84 (s, 2H), 7.57 (dd, J = 16.7, 9.1 Hz, 2H), 7.46 (d, J = 7.4 Hz, 2H), 7.42–7.15 (m, 6H), 4.93 (s, 2H), 3.69 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.02, 149.25, 148.10, 146.61, 139.70, 131.64, 130.05, 129.01, 128.89, 127.86, 127.51, 124.42, 124.17, 121.94, 118.48, 118.34, 99.42, 55.34, 54.73. HR-MS(ESI): calcd for $C_{20}H_{18}N_2O$ $[M+H]^+$ 315.1497; found: 315.1506.

4.1.1.6. 6-Chloro-*N*-benzylacridin-9-amine (5d). Yield 62%; mp 112–116 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 8.6 Hz, 2H), 8.03–7.97 (m, 2H), 7.75–7.62 (m, 1H), 7.43–7.30 (m, 6H), 7.24 (d, J = 1.9 Hz, 1H), 4.96 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 151.37, 149.66, 138.94, 136.09, 130.91, 130.50, 129.47, 129.19, 128.20, 127.88, 127.66, 124.62, 124.30, 123.79, 122.48, 116.93, 115.08, 55.12; HR-MS(ESI): calcd for $C_{20}H_{15}ClN_2$ $[M+H]^+$ 319.1002; found: 319.0993.

4.1.1.7. 6-Chloro-4-methyl-2-methoxy-*N*-benzylacridin-9-amine (5e). Yield 37%; mp 107–110 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H), 7.97 (d, J = 9.0 Hz, 1H), 7.44–7.27 (m, 7H), 7.02 (s, 1H), 4.81 (s, 2H), 3.78 (s, 3H), 2.84 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.97, 129.02, 128.82, 127.89, 127.50, 125.08, 124.16, 123.73, 97.22, 55.22, 54.83, 18.68; HR-MS(ESI): calcd for $C_{22}H_{19}ClN_2O$ $[M+H]^+$ 363.1264; found: 363.1265.

4.1.1.8. 4-Methyl-*N*-benzylacridin-9-amine (5f). Yield 39%; mp 75–77 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J = 8.6 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.99 (d, J = 7.9 Hz, 1H), 7.50–7.37 (m, 5H), 7.33 (t, J = 7.0 Hz, 1H), 7.23 (d, J = 6.0 Hz, 1H), 7.16–7.04 (m, 2H), 5.02 (s, 2H), 2.08 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.95, 137.96, 132.73, 131.99, 130.88, 129.15, 128.86, 127.83, 126.80, 124.42, 123.48, 123.23, 122.71, 122.34, 114.55, 113.51, 52.81, 18.70; HR-MS(ESI): calcd for $C_{21}H_{18}N_2$ $[M+H]^+$ 299.1548; found: 299,1544.

4.1.2. Preparation of 9-(benzyloxy)acridine (6b)

Benzyl alcohol (3.00 mmol) was dissolved in dry THF (15 mL) and then sodium hydride (3.00 mmol) was added. The mixture was stirred for 45 min at room temperature. The compound **4b** (1.00 mmol) and potassium iodide (0.25 mmol) were added. The mixture was stirred and refluxed overnight. Then the solution was evaporated. The solid was poured into water (50 mL), and extracted with ethyl acetate to give the crude product. The crude product was purified by column chromatography using petroleum ether and ethyl acetate (20:1 v/v). Yield 20%; mp >250 °C; ^{1}H NMR (400 MHz, Acetone) δ 8.29 (d, J = 8.6 Hz, 2H), 8.12 (d, J = 8.8 Hz, 2H), 7.81–7.74 (m, 2H), 7.60 (d, J = 6.6 Hz, 2H), 7.52 (ddd, J = 8.4, 6.6, 0.9 Hz, 2H), 7.45–7.34 (m, 3H), 5.42 (s, 2H). ^{13}C NMR (101 MHz, Acetone) δ 160.32, 150.62, 136.97, 130.19, 129.84, 128.63, 128.51, 128.48, 125.29, 122.70, 120.44, 78.56; HR-MS(ESI): calcd for $C_{20}H_{15}NO$ $[M+H]^+$ 286.1232; found: 286.1231.

4.1.3. Preparation of *N*-(acridin-9-yl)benzamide (7b)

To a suspension of NaH (60% in mineraloil, 80 mg) in 10 mL of DMF was added benzamide (2.00 mmol) and the mixture was stirred at room temperature for 15 min. To the resultant yellow solution was added **4b** (1.00 mmol) and the mixture was heated at reflux for 2 h. The reaction mixture was cooled and added to a mixture of 1 M HCl and ethyl acetate. The mixture was filtered and the crude product was purified by column chromatography using petroleum ether and ethyl acetate (10:1 v/v). Yield 75%; mp 166-169 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, J = 20.0 Hz, 2H), 8.05–7.78 (m, 5H), 7.68 (d, J = 42.1 Hz, 1H), 7.57–7.26 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.87, 134.27, 133.37, 132.12, 131.12, 128.60, 128.12, 127.40, 125.98, 120.94, 117.27, 106.57; HR-MS(ESI): calcd for $C_{20}H_{14}N_2O$ $[M+H]^+$ 299.1184; found: 299.1188.

4.2. Bioassay

4.2.1. Cell culture

K562 (suspension cells line) was cultured in RPMI-1640, HepG-2 (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.

4.2.2. Cell growth inhibition assay

The cells were seeded into 96-well plates 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. At 490 nm, the absorbance was measured by Multimode Detector DTX 880 (Beckman Coulter).

4.3. Biophysical evaluation

4.3.1. Materials

The measurements involving the interaction of the compound with ct DNA was carried out in Ultra-pure MilliQ water (18.2 mΩ) buffer containing 10 mM Tris and 10 mM NaCl, and adjusted to pH 7.0 with hydrochloric acid. UV–vis spectrometer was employed to check a solution of ct DNA (Sigma, purification ⩾90%) purity (A_{260}:A_{280} = 1.86) and the concentration of DNA per mole phosphate was determined optically using a molar extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm in the buffer.

4.3.2. Fluorescence emission spectra

All the emission spectra were measured on Fluorolog spectrometer. Xenon arc lamp was used as the excitation light source in the measurements of emission spectra. 5 μL of 10 mM tested compound (the final concentration was 50 μM) was incubated in Tris–HCl buffer (pH 7.0) solution, and then ct DNA was also added with the final concentration from 0 to 6 μM. The excitation wavelength was set at 420 nm.

4.3.3. Absorption spectra

UV–vis absorption spectra were all recorded on a computer-controlled Beckman Coulter DU 800 spectrophotometer by using a quartz cell having 1.0 cm pathway. 5 mM tested compounds DMSO solution (1 μL)were transferred to the quartz cell with 50 μL above buffer solution, and then a known volume (0–5) μL of 0.2 mM ct DNA was also added. The above solutions were mixed and then diluted to 100 μL with the Tris–HCl buffer (pH 7.0) solution. The solution was incubated for 5 min and then tested.

4.3.4. Circular dichroism spectra

The CD spectra of DNA oligonucleotides were carried out at room temperature by using a J-815 spectropolarimeter (JASCO) with a 0.1 cm path-length quartz cell. The CD spectrum was scanned three times and obtained by taking the average of them. The scan for buffer was subtracted from the average scan each time.

4.4. DNA topoisomerase I inhibition assay

The mixture of 100 ng of plasmid DNA pBR322 (from Takara) and 1.0 units of recombinant human DNA topoisomerase I (from Takara) was incubated without and with the prepared compounds at 37 °C for 30 min in the relaxation buffer (35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 0.01% bovine serum albumin, 2 mM spermidine, 5 mM dithiothreitol). DNA samples were then electrophoresed on a 1% agarose gel at 100 V for 25 min with a running buffer of Tris–Acetic acid–(Ethylene Diamine Tetraacetic Acid, EDTA) buffer (TAE). Gels were visualized by ethidium bromide staining under ultraviolet light.

4.5. Western blot analysis

K562 cells were cultured in 6 cm dishes, followed by treatment with compound **5c** for different concentration-periods for 48 h. Cells were centrifuged and treated with lysis buffer on ice for 0.5 h, followed by centrifugation at 20,000*g* for 10 min. Protein concentrations in the supernatant were determined using bicinchonininc acid (BCA). Lysate proteins were subjected to 15% sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to PVDF membrane (amc Biobind NT-200). After blotting, the membrane was blocked in 5% Bovine serum albumin (BSA) (Trisbuffered saline (TBS), 0.5 mM Na_3VO_4; 1 mMNaF) for 1 h, and incubated with the specific primary antibody for 2 h at room temperature. Protein bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo scientific) after hybridization with the antibody.

4.6. Flow cytometry assay

Phosphatidylserine externalization was measured by AnnexinV-FITC/PI apoptosis detection kit (Beyotime Company) according to the manufacturer's instructions.

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Supplementary data

Supplementary data (general methods and the preparation of compounds **3a**–**3f** and **4a**–**4f**; fluorescence spectroscopy and absorption spectroscopy; ^1H NMR and ^{13}C NMR spectrum; high resolution mass spectrometry) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.008.

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