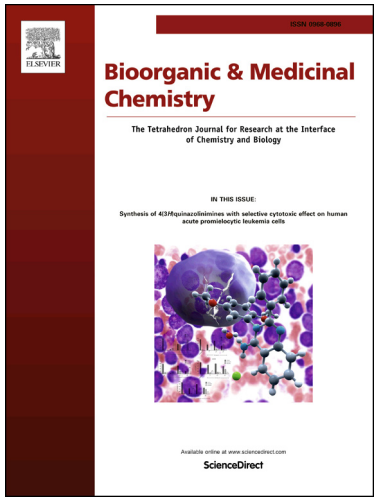

Synthesis and biological research of novel azaacridine derivatives as potent DNA-binding ligands and topoisomerase II inhibitors

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Synthesis and biological research of novel azaacridine derivatives as potent DNA-binding ligands and topoisomerase II inhibitors

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Abstract

DNA and DNA-related enzymes are one of the most effective and common used intracellular anticancer targets in clinic and laboratory studies, however, most of DNA-targeting drugs suffered from toxic side effects. Development of new molecules with good antitumor activity and low side effects is important. Based on computer aided design and our previous studies, a series of novel azaacridine derivatives were synthesized as DNA and topoisomerases binding agents, among which compound **9** displayed the best antiproliferative activity with an IC_{50} value of 0.57 μM against U937 cells, which was slightly better than *m*-AMSA. In addition, compound **9** displayed low cytotoxicity against human normal liver cells (QSG-7701), the IC_{50} of which was more than 3 times lower than *m*-AMSA. Later study indicated that all the compounds displayed topoisomerases II inhibition activity at 50 μM. The representative compound **9** could bind with DNA and induce U937 apoptosis through the exogenous pathway.

Keywords : Azaacridine derivatives, Molecular docking, DNA binding, Topoisomerase II inhibitor, Antiproliferative activity

1. Introduction

Cancer has been reported as a leading cause of death in many countries especially in less developed countries.[1] Chemotherapy, radiotherapy, and surgery are the three traditional methods for cancer, among which chemotherapy maintains the primary choice in cancer treatment. Many targets have been discovered for cancer therapy. DNA and its related enzymes (including topoisomerases, tubulin, telomerase, etc.) are one of the most effective and successful families of drug targets in clinic and laboratory studies.[2-5] Topoisomerases are universal and necessary enzymes that alter DNA topology during DNA replication, transcription, chromatin assembly by forming a reversible covalent Topoisomerases-DNA (Top-DNA) complex.[6-9] One of the mechanism of topoisomerases targeted drugs is that molecules can bind with DNA, and then inhibit the relaxation process catalyzed by DNA topoisomerases. Most of them have polycyclic conjugated structures with the ability to intercalate into DNA.[9-11]

With a conjugated planar structure, acridine and acridone derivatives have been discovered as DNA targeted drugs since World War I. Many acridine analogues have been developed to target not only DNA but also its correlated enzymes. Now some acridine compounds, such as *m*-AMSA, DACA, PZA, BRACO-19 and AS1410, have entered the clinical or preclinical stages (Figure 1).[11-16] Our research group has also developed series of acridine agents with good antitumor activity.[17-22] Compared with the huge amount of acridines, little attention has been paid on azaacridines, although limited azaacridines have been studied in pharmaceutical field such as antibacterial, antimalarial, antiparasitic, as well as antitumor agents.[11, 23]

Like acridines, benzimidazoles were also discovered to have the ability to interact with some key parts in biological systems such as DNA, motor domain of spindle kinesin, poly (ADP ribose) polymerase-1 (PARP-1), and microtubules, some of which have been developed as potent antitumor agents.[24-26] Our group had also put efforts in synthesizing several series of benzimidazoles with good antitumor activity, and found that the combination of acridine ring and benzimidazole ring by an $NHCH_2$ linker could increase the DNA binding affinity.[21, 27-28] We think that combining

azaacridine and benzimidazole ring by the $NHCH_2$ linker might offer a better therapeutic effect, because the introduction of another nitrogen in the acridine ring might increase the interaction between compounds and the targets. Therefore, the molecular docking scores (Table 1S) were preliminary investigated and illustrated that the introduction of benzimidazole group to the 9-position of azaacridine led to better DNA-Top binding ability than the corresponding 9-benzimidazole acridine. Therefore, as a part of our ongoing work in developing antitumor agents, this paper designed and synthesized two 9-benzimidazole azaacridines, as well as several 9-substituted phenyl azaacridines to generate compounds with better antitumor activity. The antiproliferative activity, the DNA binding ability, inhibition of topoisomerase activity and induction of apoptosis were also evaluated.

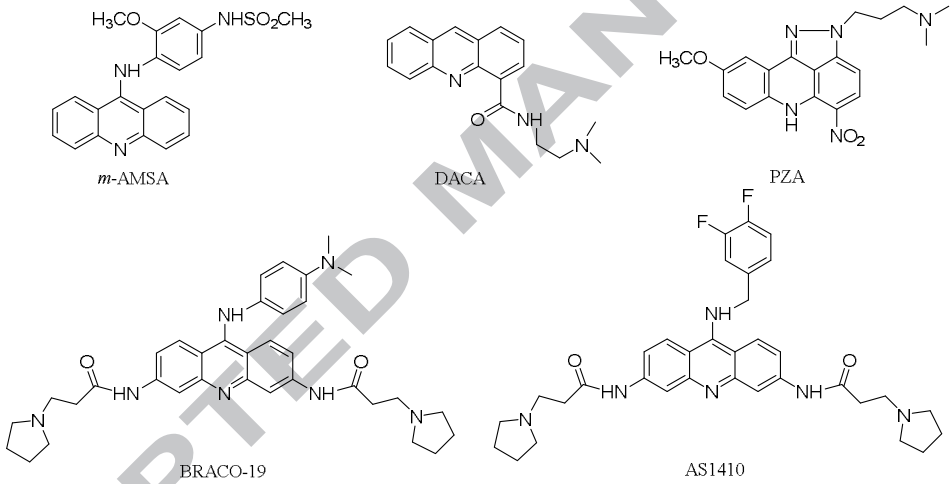


Figure 1. The typical structures of acridine analogues in clinical or preclinical stages

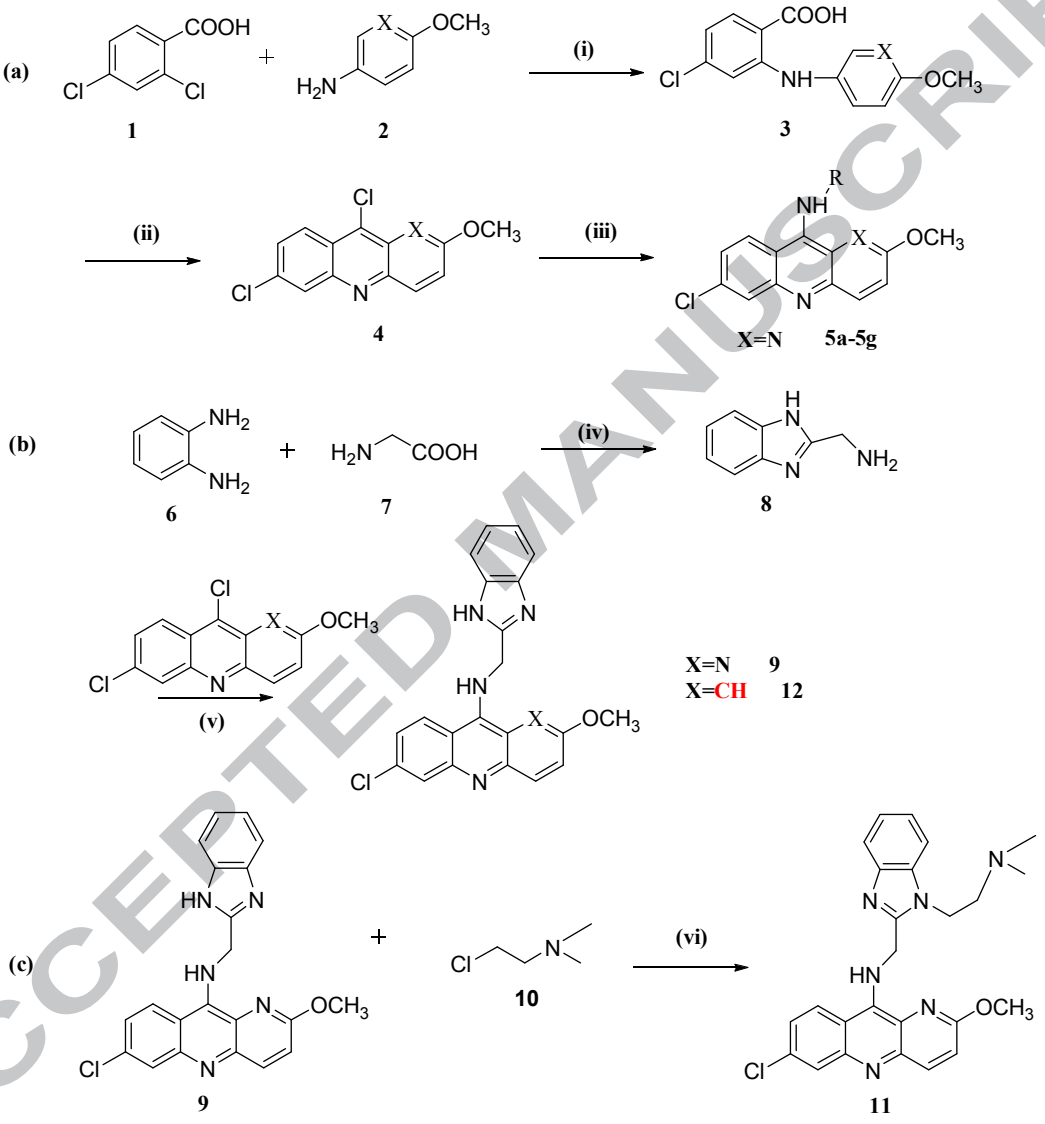
2. Results and discussion

2.1. Chemistry

The synthetic routes of target compounds **5a-5g** were carried out in Scheme 1a.[29] Anthranilic acid **3** was synthesized by using the general procedure of Ullmann reaction with Cu as a catalyst. Then **3** reacted with $POCl_3$ at 120 °C to provide the 9-chloroacridine derivative **4** in 2 hours. In the presence of phenol, compound **4** reacted with aniline derivatives to give the corresponding **5a-5g**.

As described in Scheme 1(b), 2-aminomethyl benzimidazole **8** was obtained by

commercially available *o*-phenylenediamine **6** reacting with glycine **7** in concentration hydrochloric acid under reflux conditions. Compound **8** reacted with 9-chloroacridine derivatives **4** in phenol giving compound **9** and **12**. As shown in Scheme 1(c), compound **11** was produced by a nucleophilic substitution reaction between compound **9** and 2-dimethylaminoethyl chloride.[30]



Scheme 1. Synthetic routes of the azaacridine derivatives. Reagents and conditions: (a) (i) K_2CO_3, Cu, DMF, 130 °C, overnight; (ii) $POCl_3$, 120 °C, 2 h; (iii) $CHCl_3$, ethanol, HCl, rt, overnight. (b) (iv) 6 M HCl, 100 °C, reflux, 72 h; (v) phenol, 120 °C, 2 h. (c) (vi) KOH, K_2CO_3, acetone, reflux, overnight

2.2. Antiproliferative activity in cellular assays

MTT assay, a widely used method to detect the *in vitro* cytotoxicity of drugs, was selected to evaluate the antiproliferative activity of the compounds in hand against human leukaemia K562, U937 and hepatocellular carcinoma HepG2 cells with *m*-AMSA, CPT served as the positive drugs. As the result showed in Table 1, all the compounds displayed moderate antiproliferative activity against K562 cells, among which compound **9** with benzimidazole and azaacridine scaffold displayed the best activity. The results confirmed that the introduction of benzimidazole could improve cell cytotoxicity. After introducing the dimethylaminoethyl group to the benzimidazole group, the cytotoxicity was slightly decreased. However, the water solubility was greatly increased. When R was the phenyl group, the substituent group in the phenyl ring affected the cytotoxicity greatly.

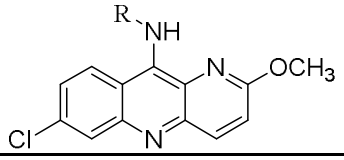
Since in medicinal chemistry study, fluorinated compounds showed remarkable record, compounds **5a-5c** were firstly synthesized to evaluate their antiproliferative activity. The result demonstrated the *ortho*-position was more preferable than *meta*- or *para*-position that was in accordance with our previous results.[17] Compound **5d** with a methoxyl group in *para*-position and **5f** with two methyl groups in *meta*- and *para*-positions displayed similar IC_{50} values. Unexpectedly, chlorinated compound **5b** presented the best antitumor activity among **5a-5g** with an IC_{50} value of 6.10 μM.

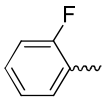
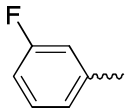
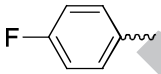
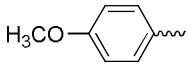
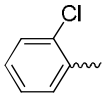
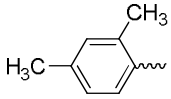
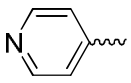
In addition, compound **5g** with a pyridine group was also synthesized, which displayed similar antiproliferative activity comparing with compounds **5b-5d**. To evaluate whether the azaacridine had the influence on the cell toxicity, compound **12** with acridine was synthesized. As shown in Table 1, **12** displayed the lowest antiproliferative activity, which indicated that azaacridine contributed much to the bioactivity.

Among all the synthesized compounds, only compounds **9** and **11** displayed good antiproliferative activity against K562, HepG2 and U937. Especially, **9** and **11** displayed nM IC_{50} values against U937, which were better or compatible to *m*-AMSA. Therefore, they were further tested against A549, Hela and QSG-7701 (Table 2). The results showed that they were more sensitive to U937. Importantly, they displayed low

activity against human normal liver cells (QSG-7701),[31] which indicated that they had the potential to be developed as antitumor agents.

Table 1. Results of the IC_{50} values of compounds **5a-5g**, **9**, **11**, **12** against K562, HepG2 and U937



Compound	R	IC_{50}(μM)[a]		
		K562	HepG2	U937
5a		10.92 ± 0.45	>20	>20
5b		15.51 ± 0.34	>20	>20
5c		16.53 ± 1.34	>20	>20
5d		13.82 ± 0.52	>20	>20
5e		6.10 ± 1.22	>20	>20
5f		14.01 ± 0.23	>20	>20
5g		13.66 ± 1.02	>20	>20
9		6.06 ± 0.89	6.62 ± 1.89	0.57 ± 0.01
11		7.59 ± 0.67	>20	0.76 ± 0.13
12		>20	>20	ND
***m*-AMSA**		0.88 ± 0.33	5.21 ± 0.74	0.61 ± 0.01

CPT	ND	4.92 ± 1.18	ND

ND : not detect

[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

Table 2. Antiproliferative activities of compounds **9**, **11** against A549, Hela and QSG-7701 cell lines

Compound	**IC_{50} (μM)[a]**		
	A549	**Hela**	**QSG-7701**
9	4.80 ± 0.18	12.62 ± 1.39	29.36 ± 3.34
11	13.12 ± 2.24	14.84 ± 1.60	21.23 ± 0.47
***m*-AMSA**	ND	19.19 ± 1.66	6.43 ± 0.05
CPT	< 1.0	3.80 ± 0.84	< 1.0

ND : not detect

[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

2.3. Molecular modeling

To confirm if the nitrogen in the azaacridine ring had an effect on the binding mode, the comparison between compounds **9** and **12** was taken based on the results of molecular docking. SYBYL-X 1.3 protocol was used to study the interaction of compounds **9** and **12** with Top-DNA complex. As seen in Figure 2A and 2B, both compounds **9** and **12** have one hydrogen bonding interaction with Arg364 which is a key residue of Top I-DNA complex. [32-33] Molecular docking results (Figure 1S) indicated that the geometry configuration of compound **9** is more similar to the typical Top I drug CPT than that of **12**. Therefore, compound **9** might display stronger Top I-DNA binding ability than compound **12**.

The docking study of compound **9** and **12** with Top II-DNA complex was shown in Figure 2C and 2D**.** Three hydrogen bonds were formed between compound **9** and Top II-DNA complex, however, only one hydrogen bond was found between compound **9** and Top II-DNA complex. The result indicated that the azaacridine may

contribute much to the binding ability, which was in accordance with the data in Table 1S.

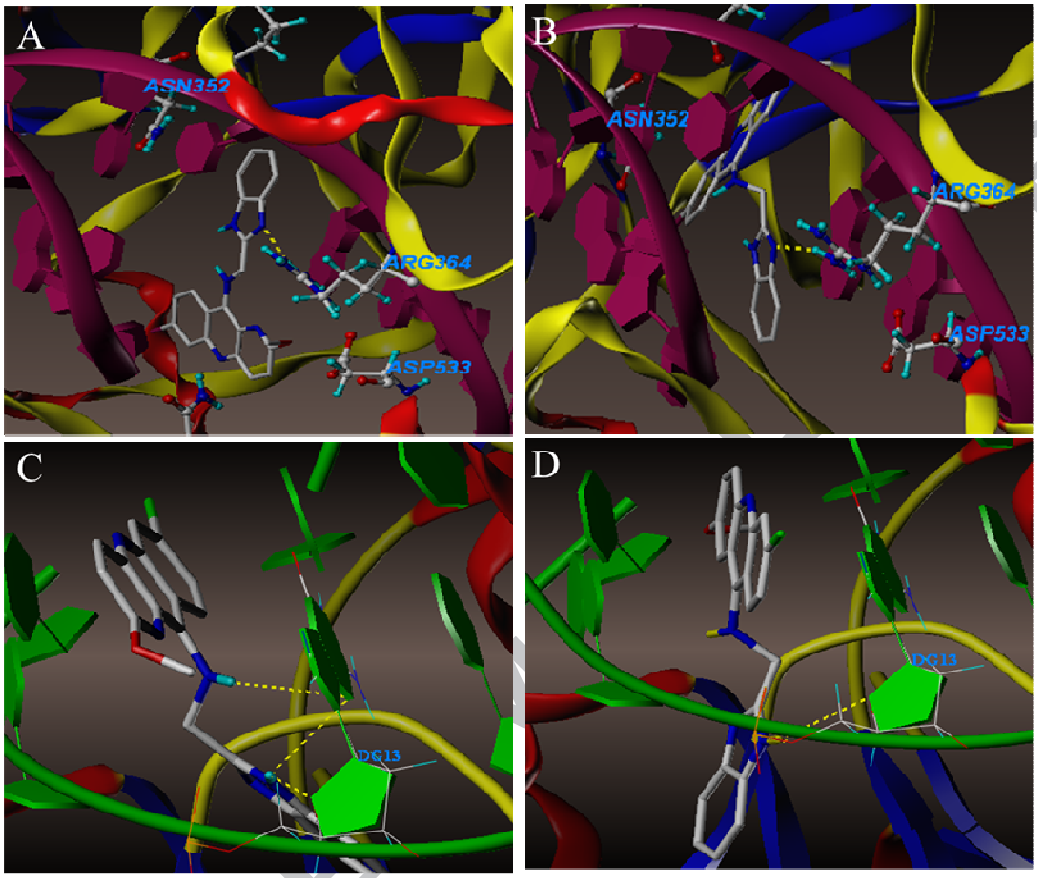


Figure 2. Molecular docking of compounds **9** and **12** with Top-DNA complex. (A) Compound **9** with Top I-DNA complex. (B) Compound **12** with Top I-DNA complex. The DNA phosphate backbone and bases are shown in purple. (C) Compound **9** with Top II-DNA complex. (D) Compound **12** with Top II-DNA complex. The DNA phosphate backbone and bases are shown in green.

2.4. DNA binding analysis

As compound **9** showed the best antiproliferative activity against several cancer cell lines, as well as low cytotoxicity against normal cells, it is necessary to explore whether the interaction with DNA contributed to the cell activity as our previous reported acridine derivatives.[17, 19, 21]

UV-visible spectral absorbance is a kind of widely and convenient measure to detect the interaction between drugs and DNA. Figure 3A displayed compound **9** had

the maximal peak at around 410 nm while DNA did not absorb light in this region. With the concentration of DNA increased, a significant decline in the absorption spectrum was observed. Beside, a slight bathochromic shift was shown, which indicated that an interaction between compound **9** and DNA occurred.[29]

In order to give a quantitative binding affinity of compound **9** with DNA, the **E_q (1)** was used to calculate the binding constant K_b where [DNA] stands for the DNA concentration, meanwhile ε_a, ε_f represent the extinction coefficient of the complex free in solution, and ε_b is the extinction coefficient of the complex when fully bound to DNA respectively. The K_b of compound **9** showed in Figure 3B indicated it had a better DNA binding capability.

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

To confirm the binding properties of compound **9**, further study of fluorescence emission spectra was employed. In Figure 3C compound **9** exhibited an emission peak around 450 nm with an excitation wavelength at 300 nm. Along with the concentration of DNA increased, the fluorescence of **9** was gradually quenched. **Formula (2)** was a classical way to calculate the quenching constant K. The quenching constant K of compound **9** was 2.34×10^5 M^{-1} which was in accordance with the absorption spectra.

$$F_0 / F = 1 + K_q [Q] \quad (2)$$

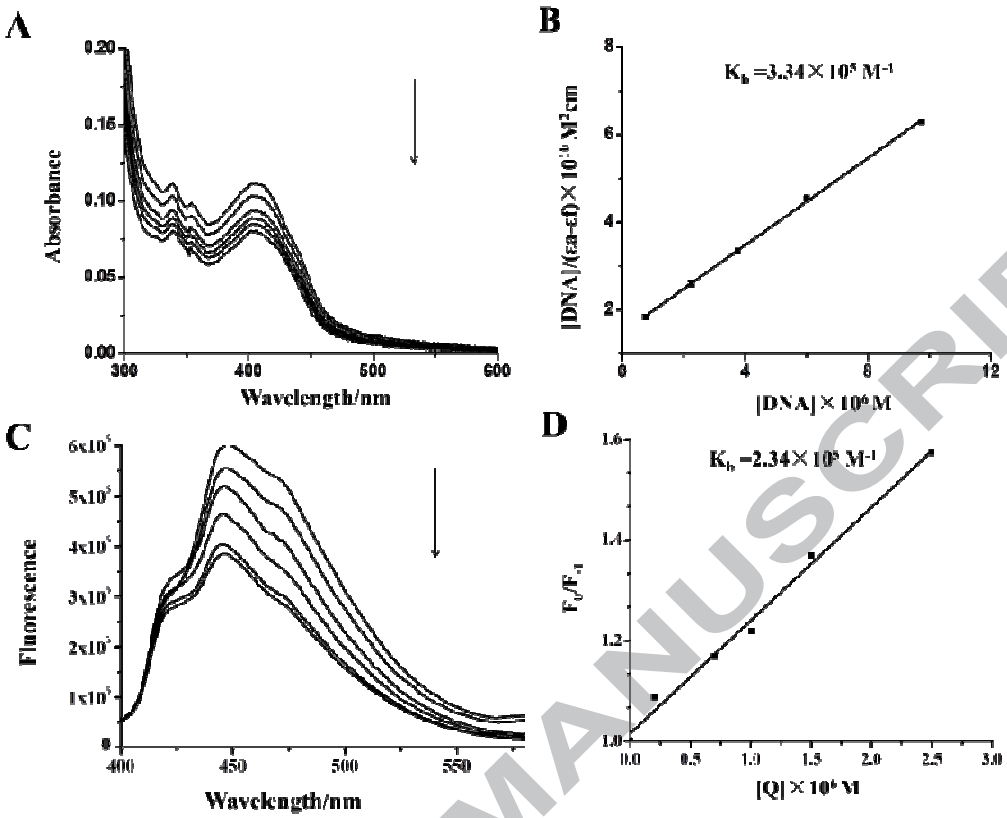


Figure 3. (A) UV-visible absorption spectra of **9** (40 μM) in the presence of increasing amounts of ct DNA ([DNA] = 0, 0.75, 2.25, 3.75, 6.00, 9.75 μM) in Tris-HCl buffer (pH 7.4). The arrow indicates the absorbance changes upon increasing DNA concentrations. (B) The plot of absorption data, [DNA], DNA concentration. (C) Spectrofluorimetric titration of **9** (40 μM) in Tris–HCl buffer (pH 7.4) by increasing the concentrations of ct DNA ([DNA] = 0, 0.2, 0.7, 1, 1.5, 2.5 μM). The arrow indicates the fluorescence emission changes upon increasing DNA concentrations. (D) The plots of the fluorescence titration, [Q], DNA concentration.

2.5. DNA Top I and Top II inhibition assay

In previous study most acridine derivatives could interact with DNA and inhibit the activity of topoisomerase.[34-35] Inhibitory activities were firstly evaluated at 100 μM and all our newly synthesized compounds exhibited apparent Top II inhibitory activity while only compound **11** showed Top I inhibitory at 100 μM (Figure 4A and 4B). Further investigation displayed that all the compounds presented Top II inhibitory activity at 50 μM (Figure 4C). To make a more intuitive and visualized

result of Top II inhibitory, the extent of remained relaxed DNA was quantitated (Figure 5). As showed in Figure 5A, most of the compounds expressed Top II inhibitory activity over 50% except compound **5a**, which displayed 28.1% Top II inhibition activity. Compounds **5b**, **5c**, **5d**, **9**, **11** displayed a comparable Top II inhibitory activity to the positive Top II drug *m*-AMSA. At 10 μM, the inhibitions of Top II by compounds **5b**, **5c**, **5e**, **5g**, **11** were over 40% and compound **11** showed the strongest Top II inhibitory activity (75.1%), which was better than *m*-AMSA (56.9%). The results suggested all the compounds may have antiproliferative activity through interacting with DNA and then inhibiting Top II.

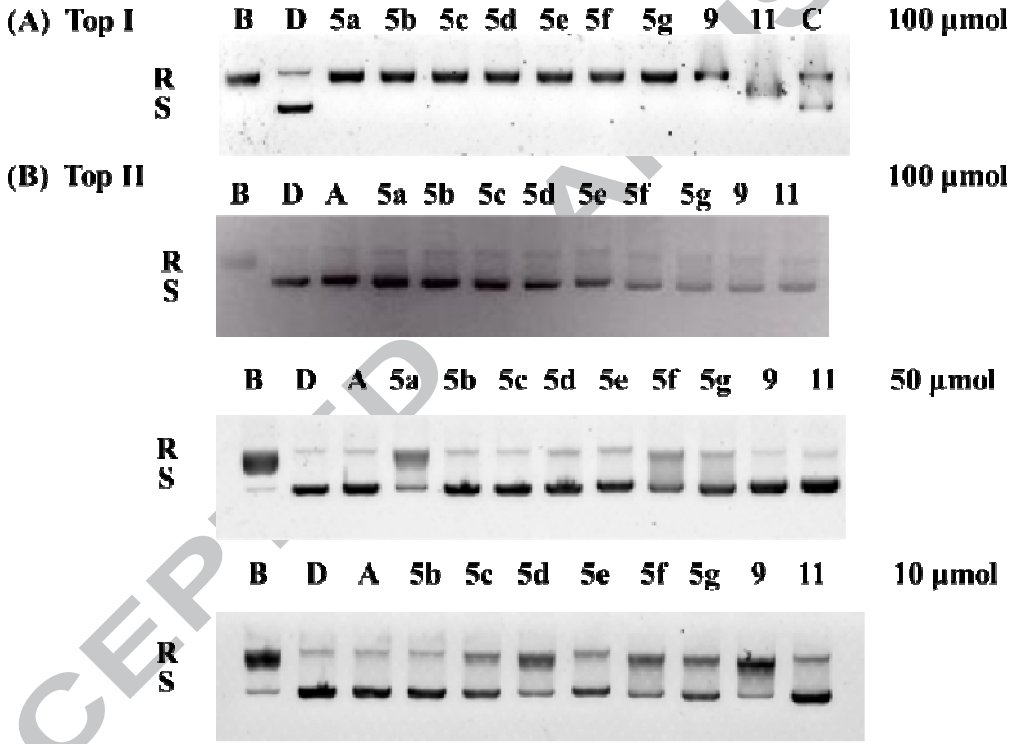


Figure 4. Top I and Top II inhibitory activities. (A) Top I: lane B, Top I + pBR322 DNA; lane D, pBR322 DNA; lane C, CPT + Top I + pBR322DNA; the others, tested compounds + Top I + pBR322 DNA. (B) Top II: lane B, Top II + pBR322 DNA; lane D, pBR322 DNA; lane A, *m*-AMSA + Top II + pBR322 DNA; the others, tested compounds + Top II + pBR322 DNA.

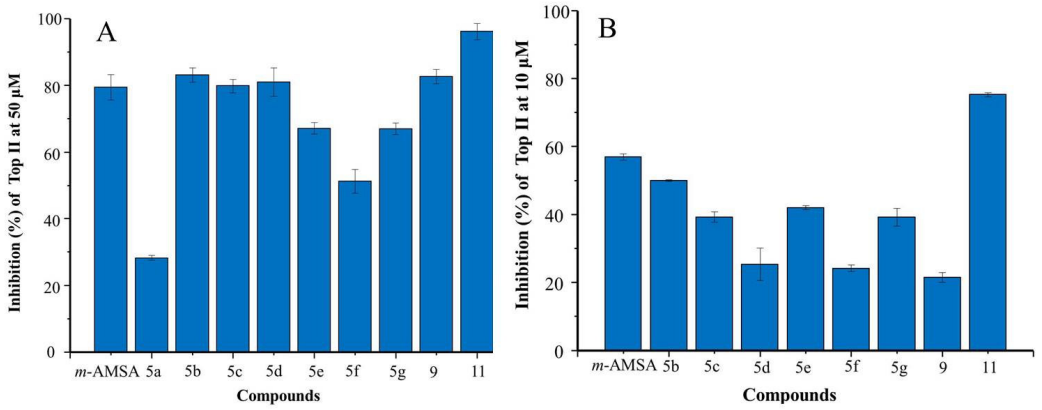


Figure 5. (A) Top II inhibitory activities at 50 μM; (B) Top II inhibitory activities at 10 μM.

2.6. Apoptosis induced by compound 9

Compound **9** could interact with DNA and inhibit the activity of Top II, which may induce apoptosis in cancer cell lines. An AnnexinV-FITC/PI binding assay using U937 cells was conducted. The results can be seen in Figure 6. Q2 is the early stage of apoptotic cells while Q3 represents the late stage of apoptotic or necrotic cells. After U937 cells were treated with compound **9** at the concentrations of 0, 1 and 2.5 μM for 48 h, we can clearly see that the percentage of apoptotic cells increased dramatically with the concentration of compound **9** increased. At 1 μM, 17.5% U937 cells were early apoptotic, and 6.48% cells were late apoptotic or necrotic. At 2.5 μM, early apoptosis and late apoptosis or necrotic cells were improved to 26.2% and 12.2%. The result indicated that compound **9** can induce U937 cell apoptosis efficiently.

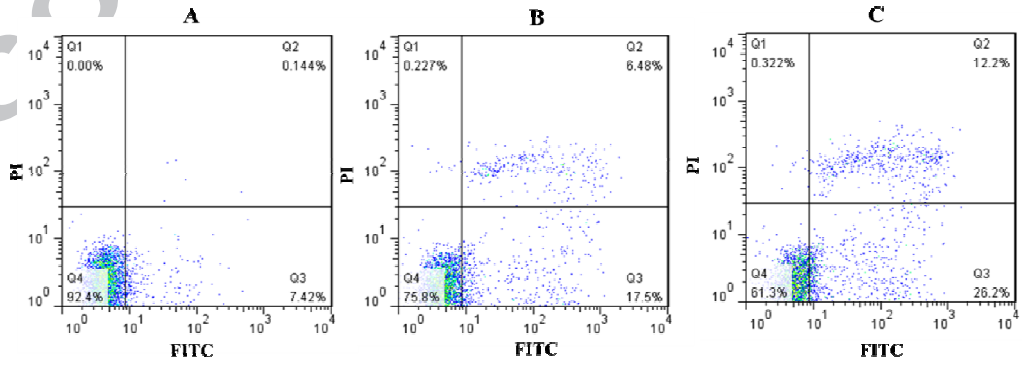


Figure 6. Flow cytometric analysis of phosphatidylserine externalization (Annexin-V binding) and cell membrane integrity (PI staining). U937 cells were treated with

compound **9** at (A) 0; (B) 1; (C) 2.5 μM respectively.

Apoptosis can be triggered by two key molecular signaling pathways: intrinsic pathway (mitochondrial pathway), extrinsic pathway (death-receptor-mediated pathway).[36] In order to investigate which pathway that cellular apoptosis was induced by compound **9**, the expressions of regulatory proteins related to apoptotic pathway such as cleaved caspase-3, cleaved caspase-7 and cleaved caspase-8 in U937 cells were monitored by immunoblotting. Caspase-8 is an initiator in death-receptor-mediated cell death pathway and caspase-3, caspase-7 show significant roles in cell apoptosis once activated.[37] In Figure 7, after treating with compound **9** at the concentrations of 0, 1 and 2.5 μM for 48 h, a significant activation of cleaved caspase-3, 7, 8 was detected. The results above indicated compound **9** induced apoptosis through an extrinsic pathway.

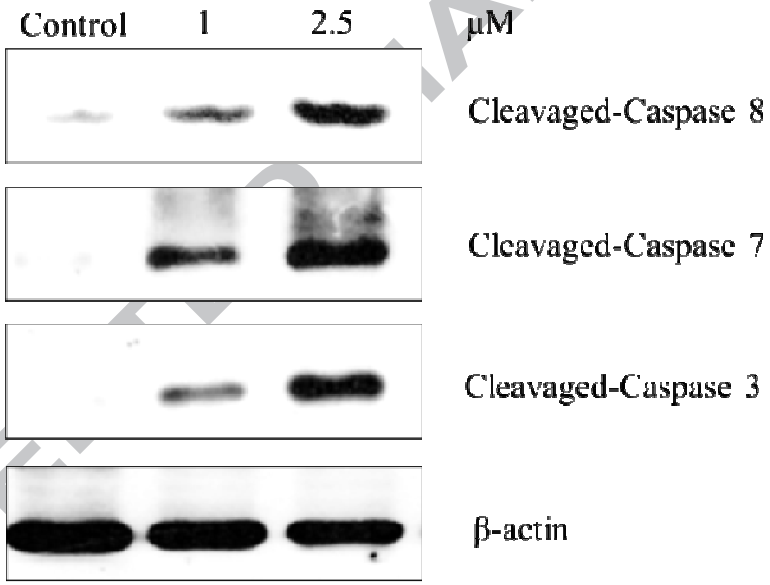


Figure 7. U937cells were treated with compound **9** at indicated concentrations and western blot analysis was used to evaluate the expression of cleavage caspase-3/7/8 in U937 after 48 h. β-actin was used as an equal loading control.

3. Conclusion

Depending on rational analysis and moleculardocking studies, a new series of 9-azaacridine derivatives with phenyl or benzimidazole moities were synthesized.

Most of the compounds displayed moderate to good cytotoxicity against K562 cells. Compounds **9** and **11** with benzimidazole showed good antitumor activity against other cancer cell lines. In addition, compounds **9** and **11** displayed lower toxicity against normal cells QST-7701 compared to *m*-AMSA. All of the compounds strongly inhibited Top II activity and only compound **11** exhibited Top I inhibitory activity at 100 μM. Most of the compounds displayed moderate to good Top II inhibitory activity at 10 μM. The representative compound **9** could strongly interact with DNA and induce U937 cells apoptosis through an extrinsic pathway. Further structural optimization of compound **9** is on the way.

4. Experimental section

4.1. Chemistry

The general preparation of compound **4** can be seen in supporting information.

4.1.1. General procedure for compound 5a-5g

A few drops of concentrated hydrochloric acid as catalysts were added dropwise to a mixture of substituted 6, 9-dichloro-2-methoyl-acridine (50 mg, 0.20 mmol) and benzenamine (0.97 mmol) in ethanol/chloroform (V/V= 4/1, 50 ml). The reaction mixture was stirred overnight at room temperature. Then the liquid was evaporated to give the crude products, which were purified by washing with ethanol twice.

4.1.1.1. 6-Chloro-2-methyl-9-(2-fluoro) phenylamino-azaacridine (**5a**) Yield 62%; mp: 281-283 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (d, J = 8.6 Hz, 1H), 8.27 (d, J = 9.1 Hz, 1H), 8.03 (s, 1H), 7.59 (d, J = 9.0 Hz, 1H), 7.53-7.23 (m, 5H), 3.42 (s, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.75, 136.02, 129.54, 128.74, 128.54, 128.42, 127.55, 127.33, 126.38, 125.42, 125.40, 124.48, 124.44, 124.35, 119.95, 116.14, 115.63, 115.44, 52.84. HRMS (ESI): $[M+H]^+$ 354.0809; found 354.0806

4.1.1.2. 6-Chloro-2-methyl-9-(3-fluoro) phenylamino-azaacridine (**5b**) Yield 65%; mp: 276-278 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (d, J=9.2 Hz, 1H), 8.25 (d, J=9.3 Hz, 1H), 8.18 (d, J=1.9 Hz, 1H), 7.60-7.47 (m, 3H), 7.33 (d, J=10.2 Hz, 1H), 7.26 (d, J=7.9 Hz, 1H), 7.19 (t, J=8.4 Hz, 1H), 3.62 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.64, 161.24, 159.41, 147.53, 146.88, 143.77, 141.55, 140.43, 133.94,

129.76, 129.35, 127.67, 127.38, 126.22, 124.86, 119.33, 118.30, 115.56, 108.05, 107.86, 106.61, 106.41, 53.22. HRMS (ESI): $[M+H]^+$ 354.0809; found 354.0805

4.1.1.3. 6-Chloro-2-methyl-9-(4-fluoro) phenylamino-azaacridine (**5c**) Yield 59%; mp: 268-270 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 (d, J=9.2 Hz, 1H), 8.13 (d, J=1.8 Hz,1H), 8.05 (s, 1H), 7.65-7.48 (m, 4H), 7.39 (t, J=8.7 Hz, 2H), 3.75 (s,3H). ^{13}C NMR (101MHz, DMSO-d_6) δ 159.27, 159.07, 156.90, 147.32, 143.19, 142.93, 142.79, 140.25, 139.95, 133.90, 128.33, 127.30, 127.14, 126.30, 124.00, 123.16, 123.09, 119.09, 116.73, 115.28, 115.06, 53.22. HRMS (ESI): $[M+H]^+$ 354.0809; found 354.0806.

4.1.1.4. 6-Chloro-2-methoyl-9-(4-methoyl) phenylamino-azaacridine (**5d**) Yield 62%; mp: 285-287 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.16 (s, 1H), 8.36 (d, J=9.2 Hz, 1H), 8.05 (d, J=2.1 Hz, 1H), 7.84 (s, 1H), 7.60 (d, J=9.1 Hz, 1H), 7.49-7.35 (m, 3H), 7.12 (d, J=8.8 Hz, 2H), 3.92 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.57, 158.11, 158.08, 137.91, 133.43, 133.34, 127.45, 126.66, 126.62, 126.59, 124.07, 121.78, 114.68, 112.78, 55.41, 54.14. HRMS (ESI): $[M+H]^+$ 366.1009; found 366.1001.

4.1.1.5. 6-Chloro-2-methoyl-9-(2-chloro) phenylamino-azaacridine (**5e**) Yield 69%; mp: 289-291 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 (d, J=9.2 Hz, 2H), 8.17 (s, 1H), 7.74-7.59 (m, 3H), 7.59-7.49 (m, 2H), 7.45 (t, J=7.2 Hz, 1H), 3.48 (s, 3H). ^{13}C NMR (101MHz, DMSO-d_6) δ 158.63, 136.55, 133.94, 129.36, 128.79, 128.48, 128.33, 127.50, 127.16, 126.93, 125.03, 124.04, 124.02, 119.79, 52.86. HRMS (ESI): $[M+H]^+$ 370.0514; found 370.0507.

4.1.1.6. 6-Chloro-2-methyl-9-(2,4-dimethyl) phenylamino-azaacridine (**5f**) Yield 53%; mp：>300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (s, 1H), 7.94 (s, 1H), 7.77 (s,1H), 7.37-7.35 (d, J=7.8 Hz, 1H), 7.28-7.26 (d, J=9.1 Hz, 1H), 7.20 (s, 1H), 7.03 (m, 2H), 3.81 (s, 3H), 2.27 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.65, 151.90, 139.54, 138.94, 137.75, 136.30, 134.49, 131.52, 131.28, 129.22, 127.59, 126.96, 126.56, 125.27, 124.57, 122.36, 118.31, 54.11, 20.75, 17.58. HRMS (ESI): $[M+H]^+$ 364.1217; found 364.1211

4.1.1.7. 6-Chloro-2-methyl-9-(4-pyridine) amino-azaacridine (**5g**) Yield 67%; mp:

245-247 °C; ^{1}H NMR (400 MHz, DMSO-d_6/D_2O) δ 8.17-8.10 (m, 4H), 7.98 (s, 1H), 7.50-7.48 (d, J=9.2Hz, 1H), 7.27-7.24 (d, J=9.2, 2H), 6.79-6.78 (m, 2H), 3.54 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6/D_2O) δ 171.98, 159.64, 157.24, 148.01, 139.95, 134.41, 130.48, 129.21, 126.36, 125.69, 120.53, 119.45, 118.66, 115.15, 112.24, 53.07. HRMS (ESI): $[M+H]^+$ 336.0788; found 336.0857.

4.1.2. Preparation of compound 9, 12

A mixture of 6,9-dichloro-2-methoyl-anzacridine (278 mg, 1 mmol) and benzimidazoles (220 mg, 1 mmol) was heated in phenol under 120 °C for 2 hours with nitrogen protection. Then the mixture was poured into ethyl acetate to give yellow precipitates, which were purified by washing with ethyl acetate twice.

4.1.2.1. 6-Chloro-2methyl-9-((1H-benzo[d]imidazol-2-yl)methyl)-azaacridine (**9**) Yield 73%; mp: 289-291 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51-8.48 (d, J=14.4 Hz 1H), 8.37 (s, 1H), 8.13-8.11 (d, 1H), 7.92 (s, 1H), 7.47-7.42 (m, 3H), 7.22-7.20 (d, J=8.8 Hz, 1H), 7.12-7.10 (m, 2H), 3.81 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 157.82, 153.67, 147.62, 146.32, 143.49, 140.36, 140.33, 140.28, 133.72, 127.24, 126.19, 125.45, 123.24, 118.35, 115.08, 53.37, 44.72. HRMS (ESI): $[M+H]^+$ 390.1043; found 390.1115.

4.1.2.2. 6-Chloro-2methyl-9-((1H-benzo[d]imidazol-2-yl)methyl)-acridine (**12**) Yield 22%; mp: 237-238 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63-8.61 (d, J = 8.1 Hz, 2H), 8.03 (s, 1H) 7.94-7.89 (m, 2H), 7.74-7.73 (d, J = 4.8 Hz, 1H), 7.62-7.53 (m, 2H), 5.54 (s, 2H) 3.88 (s, 3H) ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.13, 155.86, 139.16, 138.90, 134.79, 134.06, 127.93, 126.86, 123.94, 120.43, 117.32, 112.49, 109.67, 103.22, 56.28. HRMS (ESI): $[M+H]^+$ 389.1169; found 389.1163.

4.1.3. Preparation of compound 11

KOH (30 mg) and K_2CO_3 (60 mg) as bases were added to an acetone suspension containing compound **9** (100 mg, 0.31 mmol) and 2-Dimethylaminoethyl chloride hydrochloride (45 mg, 0.31 mmol), which were refluxed overnight. After cooling to room temperature, water was added into the mixture under ice-water bath. Purified yellow solids were filtrated after washing by water once.

4.1.3.1. 6-Chloro-2methyl-9-((1-Dimethylaminoethyl-benzo[d]imidazol-2-yl)met

hl)-azaacridine (**11**) Yield 43% ; mp: 182-184 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (s, 1H), 8.25 (s, 1H), 8.18-8.16 (m, 1H), 8.05-8.03 (m, 1H), 7.39-7.36 (m, 1H), 7.33-7.31 (m, 1H), 7.30-7.26 (m, 1H), 7.19-7.17 (m, 1H), 5.54 (s, 1H), 4.24-4.22 (m, 2H), 4.21 (s, 1H), 2.67-2.64 (m,2H), 2.21 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.97, 150.95, 148.09, 146.43, 142.28, 141.78, 139.62, 134.95, 134.49, 127.84, 126.94, 124.14, 123.50, 122.38, 121.81, 119.28, 118.11, 114.45, 108.73, 57.96, 53.32, 45.26, 44.46, 41.90, 29.10. HRMS (ESI): $[M+H]^+$ 461.1778; found 461.1853.

4.2. Cell proliferation assay

Cell proliferation assays based on MTT were performed with 6 cell lines. Briefly, cells were seeded in 96-well plates at a density of 5-10×10^3 cells per well with incubation overnight in a 5% CO_2 incubator at 37 °C, the growth medium in each well was then added with 50 μL of fresh medium containing graded concentrations of compounds to be tested or equal DMSO and incubated continuously for 48 h. Then 15 μL MTT solution (5 mg/mL) was added to each well, and the cells were incubated for additional 4 h. The MTT-formazan crystals were dissolved in 100 mL of DMSO, the absorbance of each well was measured at 490 nm using an automatic ELISA reader system (TECAN, CHE).

4.3. UV–visible absorption spectra

All the emission spectra were measured on a computer-controlled Beckman Coulter DU 800 spectrophotometer. The pathway of the cell is 1.0 cm. The solution of tested compound **9** (5 mM in DMSO, 8 μL) and the buffer above were transferred to the quartz, then a range of ct DNA solution (2.5 mM in Tris–HCl) was mixed with the solution in the cell with different final concentration ([DNA] = 0, 0.75, 2.25, 3.75, 6.00, 9.75 μM). The incubating time before testing was 5 min.

4.4. Fluorescence emission spectra

The emission spectra were carried out on Fluorolog spectrometer. The solution of tested compound **9** (5 mM in DMSO, 8 μL) and the buffer above (2 mL) were transferred to the quartz with the final concentration at 2 μM, then a range of ct DNA solution (2.0 mM in Tris–HCl) was mixed with the solution in the cell with the final concentration ([DNA] = 0, 0.2, 0.7, 1, 1.5, 2.5 μM). The incubating time before

testing was 5 min.

4.5. DNA Top I and Top II inhibition assay

The inhibition of compounds to Top I and Top II were determined by assessing the relaxation of supercoiled pBR322 plasmid DNA. The assay was performed in a final volume of 20 μL reaction volume containing 500 ng of supercoiled DNA pBR322 (Takara Biotechnology, Japan) and 1 unit of human Top I (Takara Biotechnology, Japan) or Top II (USB Corp, USA) with or without our compounds in the reaction buffer. The reaction mixtures were incubated at 37 °C or 10-15 min and terminated by adding 4 mL of DNA loading buffer. Electrophoresis was performed on a 1% agarose gel at 90 V for 25 min in TAE buffer. Then stained gels for 10 min with ethidium bromide (2.5 mg/mL) and destained in water for 5 min. DNA bands were visualized with UV light. CPT (Sigma, USA) was used as a positive control as Top I inhibitor and *m*-AMSA (Sigma, USA) were used as a Top II inhibitor, respectively.

4.6. Flow cytometry assay

The Phosphatidylserine externalization was determined with the scheme which is designed as the instructions of the manufacturer by AnnexinV-FITC/PI apoptosis detection kit.

4.7. Western blot analysis

U937 cells were cultured in 6 cm dishes, and then compound **9** was added with the final concentration at 1 μM, 2.5 μM independently, and the resulting solutions were incubated for 48 h. Then the cells were centrifuged and was dialyzed with lysis buffer at 0 °C for 0.5 h, followed by centrifugation at 20,000 g for 10 min. Protein concentrations in the supernatant were determined using bicinchoninic acid (BCA). Lysate proteins were subjected to 12% 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% milk for 1 h, and incubated with the specific primary antibody for overnight at 4 °C. Protein bands were detected using the BIO-RAD Gel Doc XR after hybridization with the antibody.

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Supplementary data

Supplementary data (Molecular modeling of compound **9**, **12** and **CPT** with Top I -DNA complex, synthesis of compound **4**, ^{1}H NMR and ^{13}C NMR spectra and high resolution mass spectrometry of compounds **5a**-**5g**, **9**, **11** and **12**, associated with this article can be found in the online version.

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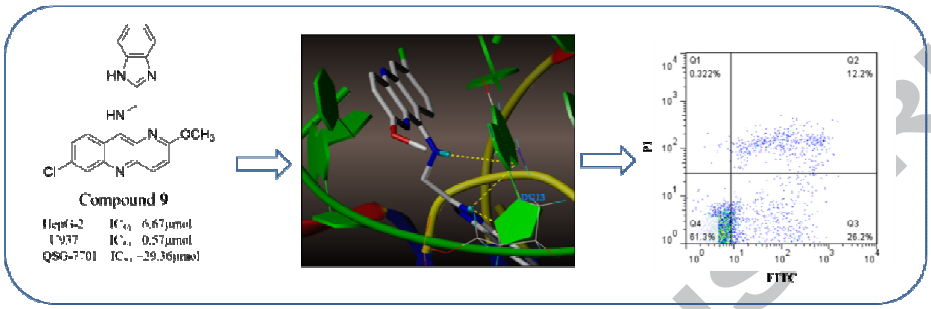
Synthesis and biological research of novel azaacridine derivatives as potent DNA-binding ligands and topoisomerase II inhibitors



A series of azaacridine derivatives were synthesized and evaluated as good antiproliferative inhibitors. Compared to *m*-AMSA, the typical compound **9** showed comparable IC_{50} values against cancer cells (HepG-2 and U937 cells) while displayed lower cytotoxicity against human normal liver cells (QSG-7701).

Highlights

- A series of azaacridine derivatives were synthesized.
- Most of the compounds displayed good antiproliferative activity.
- The typical compound **9** showed low cytotoxicity against human normal liver cells.
- All the compounds displayed good topoisomerase II inhibition activity.
- Compound **9** induced apoptosis through the exogenous pathway.