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Original article

# 12-*N*-Methylated 5,6-dihydrobenzo[*c*]acridine derivatives: A new class of highly selective ligands for *c-myc* G-quadruplex DNA

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ABSTRACT

12-*N*-Methylated and non-methylated 5,6-dihydrobenzo[*c*]acridine derivatives were designed and synthesized as new series of *c-myc* G-quadruplex binding ligands. Their interactions with *c-myc* G-quadruplex were evaluated using fluorescence resonance energy transfer (FRET) melting assay, circular dichroism (CD) spectroscopy, surface plasmon resonance (SPR), polymerase chain reaction (PCR) stop assay, and molecular modeling. Compared with the non-methylated derivatives, 12-*N*-methylated derivatives had stronger binding affinity and stabilizing ability to *c-myc* G-quadruplex structure, and could more effectively stack on the G-quartet surface. All these derivatives had high selectivity for *c-myc* G-quadruplex DNA over duplex DNA. The reverse transcription (RT) PCR assay showed that compound **21c** could down-regulate transcription of *c-myc* gene in Ramos cell line containing NHE $III_1$ element, but had no effect in CA46 cell line with NHE $III_1$ element removed.



## 1. Introduction

Guanine-rich sequences can self-associate into planar guanine quartets (G-quartets) that stack on each other to form unusual structures called G-quadruplexes [1,2]. G-Quadruplexes can be stabilized by cations (preferring $K^+$) and widely distribute in telomeric regions [3,4] and gene promoters such as *c-myc* [5,6], *c-kit* [7], *bcl-2* [8], and *VEGF* [9]. The formation or stabilization of G-quadruplexes in these regions may result in a series of biological processes such as inhibition of telomerase [10], prevention of telomere elongation [11], avoidance of chromosomal alignment and recombination [3], and down-regulation of gene expression [12]. Ligands that can selectively bind to and stabilize G-quadruplex can interfere with telomere maintenance [13] or regulate gene expression [14,15], therefore are promising lead compounds for cancer treatment [16,17]. Several series of *c-myc* G-quadruplex ligands have been developed, including macrocyclic [18–20], large coplanar [14,18,20–22], and flexible molecules [20,23–25], in which some are alkaloid derivatives or their methylated products, and the latter showed better potency.

Acridine is a fused polycyclic aromatic molecule, which is a main scaffold in some natural acridine alkaloids [26]. Its derivatives such as amasacrine and quinacrine have been originally used as effective DNA-intercalating agents [27]. Recent years, acridine derivatives have been modified as telomeric [28–34] or *c-myc* [18,20] G-quadruplex ligands, in which some excellent ligands have been discovered, such as BRACO-19 [20], BOQ1 [32], and RHPS4 [35]. Although most of these ligands have shown high telomerase inhibitory activity ($IC_{50} < 1.0$ μM), they have exhibited moderate stabilizing ability ($\Delta T_m \sim 10$ °C) and low selectivity to G-quadruplex structures. It should be noted that an effective G-quadruplex binding ligand should have not only good bioactivity, but also high selectivity, in order to avoid acute toxicity and intolerable side effects in normal tissues.

With this aim in mind and based on the previous studies, we designed and synthesized a new class of crescent-shaped 5,6-dihydrobenzo[*c*]acridine derivatives (Fig. 1) as *c-myc* G-quadruplex ligands. Unlike the previous completely coplanar acridine molecules, our new acridine derivatives have a saturated C–C bond at the 5,6-position forming a slightly-twisted planar ring scaffold with non-methylated (Fig. 1A) and methylated (Fig. 1B) structures. The rationale for designing these derivatives are as follows: At first, it is interesting to know whether these partially-saturated acridine derivatives can act as G-quadruplex binding ligands, with good

*Abbreviations:* FRET, fluorescence resonance energy transfer; CD, circular dichroism; SPR, surface plasmon resonance; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; RT, reverse transcription; MTT, methyl thiazolyl tetrazolium.

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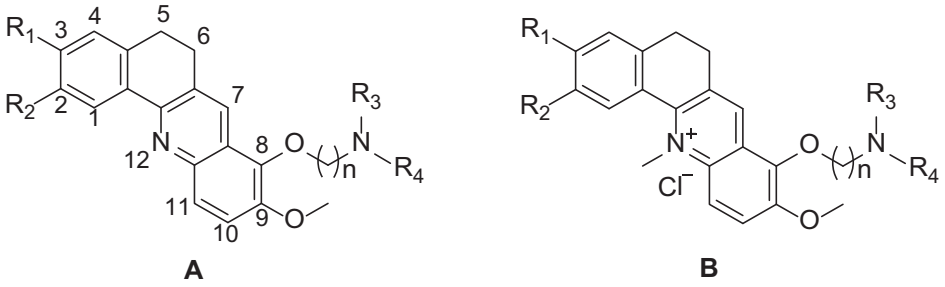



**Fig. 1.** Scaffold of 5,6-dihydrobenzo[*c*]acridine derivatives.

selectivity toward G-quadruplex DNA over duplex DNA. Secondly, some previous studies [35,36] have shown that the methylation of the nitrogen atom in the center of the ligands could strongly enhance their binding and stabilizing activity toward G-quadruplex, therefore, it is interesting to know whether the methylation of our new acridine derivatives can also increase their interactions with G-quadruplex. Thirdly, some studies have shown [32,37] that the fused crescent-shaped planar structure could offer effective π-orbital stacking on the G-quadruplex, therefore it is interesting to know whether our nearly planar crescent-shaped ligands can also effectively interact with G-quadruplex through π-orbital stacking. Lastly, it is interesting to know whether the 1,3-dioxole ring is better for the ligands to interact with G-quadruplex than the two methoxy groups. Therefore, we synthesized a series of 5,6-dihydrobenzo[*c*] acridine derivatives (Fig. 1), and studied their interactions with *c-myc* G-quadruplex DNA through FRET-melting, CD spectroscopy, SPR assay, PCR-stop assay, RT-PCR assay, and molecular modeling.

## 2. Chemistry

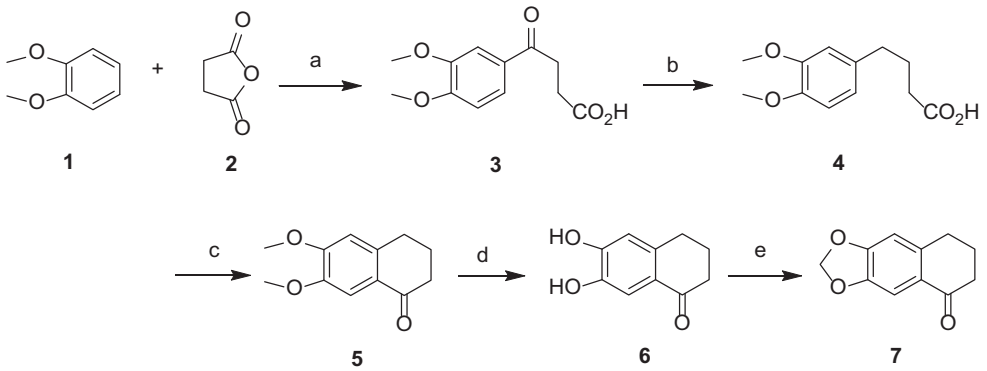
The 5,6-dihydrobenzo[*c*]acridine derivatives were synthesized as follows: 1,2-dimethoxybenzene **1** was acylated with dihydrofuran-2,5-dione **2** using $AlCl_3$ as catalyst in nitrobenzene at 0–60 °C to afford compound **3**, which was then deoxidized [38] and intra-cyclized to afford intermediate **5**. The intermediate **5** was demethylated with 48% HBr to give compound **6**, which was cyclized with dibromomethane under KF to yield intermediate **7** (Scheme 1) [39].

Compound **8** was synthesized by following a previously reported procedure [40], which then had the oxidation of its formyl group and the reduction of its nitro group to yield intermediate **9** (Scheme 2). Next the intermediate **5** or **7** was condensed with **9** in phosphorus oxychloride under reflux condition [41], and the product **10** or **11** had dechlorination, hydrolysis of sulfonic group, and alkylation with dibromoalkanes to afford compound **12**–**14**. Then the bromine atom was replaced with amine to yield final products **15a**, **15b**, **16a**, **17a**, and **17c**. Compound **12**–**14** could also be methylated with methyl triflate on the nitrogen atom [42], and then the bromine atom was replaced with amine, and lastly $OTf_3^-$ was exchanged into $Cl^-$ using anion exchange resin [43] to yield final products **20a**–**e**, **21b**, and **21c** (Scheme 2).

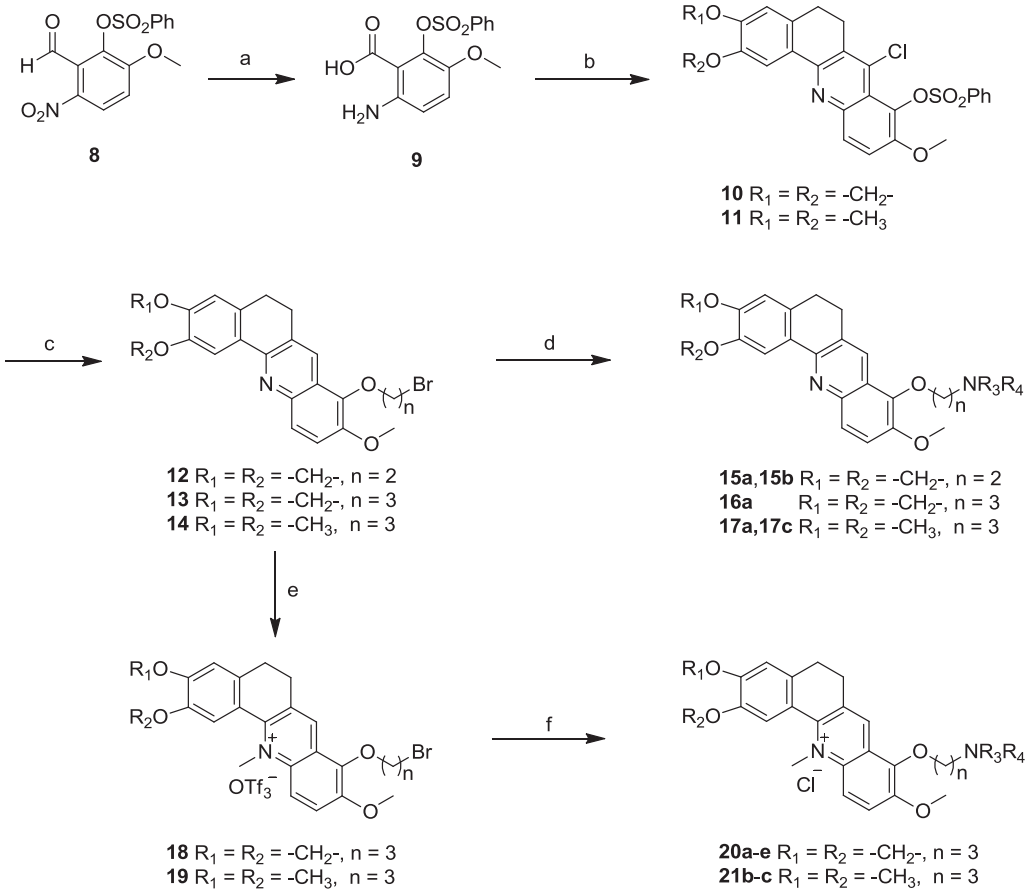
## 3. Result and discussion

### 3.1. Stabilizing ability and selectivity studies with FRET

The stabilizing ability of 5,6-dihydrobenzo[*c*]acridine derivatives on *c-myc* G-quadruplex DNA was evaluated with FRET-melting experiments [44]. The *c-myc* gene FPu18T (5′-FAM-$AG_3TG_4AG_3TG_4$-TAMRA-3′) was used as the G-quadruplex DNA model. The FRET-melting data (Table 1) showed that the $\Delta T_m$ values for all the compounds were in a wide range from 5.3 °C to 25.4 °C. The FRET results indicated that the methylated derivatives ($\Delta T_m$ values of 11.6–25.4 °C) had higher stabilizing ability than the non-methylated ones ($\Delta T_m$ values of 5.3–11.2 °C), which suggested that the central positive charge made an important contribution for the methylated derivatives to interact with *c-myc* G-quadruplex, and implied the nitrogen atom at the 12-*N*-position had weak tendency of protonation. Among all the methylated derivatives, **21b** and **21c** with the 2,3-dimethoxy moiety had the highest potency to stabilize the *c-myc* G-quadruplex DNA with $\Delta T_m$ values of 25.4 °C and 23.2 °C respectively, while their counterparts **20b** and **20c** with the 1,3-dioxole ring had lower affinity with the $\Delta T_m$ values of 22.2 °C and 21.4 °C, respectively, indicating the 1,3-dioxole ring was unfavorable for the interaction of 5,6-dihydrobenzo[*c*]acridine derivatives with G-quadruplex. Similar results were obtained for




**Scheme 1.** Synthesis of intermediates **5** and **7**. Reagents and conditions: (a) $AlCl_3$/nitrobenzene, 0–60 °C, 3 h; (b) $Et_3SiH/CF_3CO_2H$, reflux, 2 h; (c) PPA/DCM, reflux, 2 h; (d) 48% HBr, 125 °C, 4 h; (e) $CH_2Br_2$, KF/DMF, 140 °C, 6 h.




| Compounds | a | b | c | d | e |
|---|---|---|---|---|---|
| $NR_3R_4$ | | | | | |

**Scheme 2.** Synthesis of compounds **15a**, **15b**, **16a**, **17a**, **17c**, **20a**–**e**, **21b**, and **21c**. Reagents and conditions: (a) (i) $KMnO_4$/acetone, rt, 2 h; (ii) Fe, acetic acid/ethanol/water, rt, 4 h; (b) **5** or **7**, $POCl_3$, reflux, 8 h; (c) (i) $H_2$,10% Pd/C, DMF/ethanol, 60 °C, 3 h; (ii) NaOH, dioxane/water, 100 °C, 2 h; (iii) dibromoalkanes, $K_2CO_3$/acetonitrile, 60 °C, 3 h; (d) amine, $K_2CO_3$/acetonitrile, 60 °C, 3–6 h; (e) methyl triflate/toluene, rt, 1 h; (f) (i) amine/DCM, cat. KI, 2 day; (ii) dowex (Cl).

those non-methylated compounds, and the compounds **17a** and **17c** with two methoxy groups had the highest $\Delta T_m$ values. For methylated 5,6-dihydrobenzo[*c*]acridine derivatives (**20a**–**e**, **21b**, and **21c**) with amino side chains at their 8-position, their stabilizing ability appeared to be mostly similar. The only exception is ligand **20e** with a cyclohexylamino group at the end of its side chain, which might be due to its weak protonation ability because of the steric hindrance of cyclohexylamino group.

The melting temperature of FPu18T treated with various concentrations (0.1–6.0 μM) of **21c** was also monitored with FRET-melting assay as shown in Fig. 2, which showed that the melting temperature of *c-myc* G-quadruplex was concentration-dependent.

The interaction of all the derivatives with duplex DNA was also examined with the FRET-melting experiments. The oligomer F10T (5′-FAM-TATAGCTA-TA-HEG-TATAGCTATA-TAMRA-3′) that could self-associate into a hairpin structure was used as a duplex DNA model [24]. As shown in Table 1, the $\Delta T_m$ values for F10T treated with all the derivatives were very low, indicating that all compounds exhibited poor ability to stabilize the duplex DNA and thus had good selectivity to G-quadruplex DNA over duplex DNA.

Furthermore, the selectivity of the derivatives for G-quadruplex DNA over duplex DNA was also evaluated by using FRET-based competition assay, and the binding affinity of the ligands to G-quadruplex was challenged by non-fluorescent complementary DNA (self-complementary ds26 DNA: 5′-GTTAGCCTAGCTTAAGC-TAGGCTAAC-3′) [24,45]. As shown in Fig. 3, in the presence of 15 or 50 folds competitor of ds26, the $\Delta T_m$ values of FPu18T raised by the ligands were only slightly affected, showing that the complementary DNA had negligible effect on the binding between the ligands and G-quadruplex DNA.

### 3.2. Binding affinity and selectivity studies with SPR

Being surprised to the FRET results of the 5,6-dihydrobenzo[*c*] acridine derivatives interacting with the *c-myc* G-quadruplex DNA, we further studied the binding affinity of these acridine derivatives on *c-myc* G-quadruplex DNA using surface plasmon resonance (SPR) methods with biotinylated *c-myc* DNA and duplex DNA attached to a streptavidin-coated sensor chip [46,47]. The compounds with a range of concentrations were injected simultaneously to the

**Table 1**
G-Quadruplex DNA and duplex DNA stabilization temperatures ($\Delta T_m$) obtained from FRET-melting and equilibrium binding constants ($K_D$) measured with SPR.

| Compound | *n* | $R_1$, $R_2$ | $NR_3R_4$ | FRET ($\Delta T_m$/°C) | | SPR ($K_D$/μM) | |
|---|---|---|---|---|---|---|---|
| | | | | FPu18T[a] | F10T[b] | G4[c] | Duplex[d] |
| **15a** | 2 | $-OCH_2O-$ | Diethylamino | 6.3 ± 0.5 | 1.6 ± 0.3 | 2.6 ± 0.3 | – |
| **15b** | 2 | $-OCH_2O-$ | Pyrrolidino | 5.3 ± 0.4 | 1.0 ± 1.0 | 1.4 ± 0.2 | – |
| **16a** | 3 | $-OCH_2O-$ | Diethylamino | 6.2 ± 0.4 | 1.0 ± 0.7 | 3.0 ± 0.6 | – |
| **17a** | 3 | $CH_3O-$, $CH_3O-$ | Diethylamino | 11.2 ± 1.7 | 0.4 ± 0.0 | 4.4 ± 0.7 | – |
| **17c** | 3 | $CH_3O-$, $CH_3O-$ | Piperidino | 11.0 ± 1.3 | 1.0 ± 1.0 | 2.8 ± 0.5 | – |
| **20a** | 3 | $-OCH_2O-$ | Diethylamino | 21.8 ± 1.1 | 1.2 ± 0.8 | 0.6 ± 0.1 | – |
| **20b** | 3 | $-OCH_2O-$ | Pyrrolidino | 22.2 ± 0.6 | 1.1 ± 0.8 | 0.4 ± 0.1 | – |
| **20c** | 3 | $-OCH_2O-$ | Piperidino | 21.4 ± 1.4 | 0.5 ± 0.3 | 0.6 ± 0.4 | – |
| **20d** | 3 | $-OCH_2O-$ | *N*-methylpiperazino | 19.0 ± 1.6 | 0.5 ± 0.2 | 0.8 ± 0.2 | – |
| **20e** | 3 | $-OCH_2O-$ | Cyclohexylamino | 11.6 ± 1.2 | 0.9 ± 0.8 | 1.2 ± 0.2 | – |
| **21b** | 3 | $CH_3O-$, $CH_3O-$ | Pyrrolidino | 25.4 ± 0.7 | 1.4 ± 0.0 | 0.6 ± 0.1 | – |
| **21c** | 3 | $CH_3O-$, $CH_3O-$ | Piperidino | 23.2 ± 1.6 | 0.8 ± 0.2 | 0.6 ± 0.1 | – |

[a] $\Delta T_m$ values for incubation of 0.2 μM FPu18T with and without 2.0 μM compound and 0.2 mM KCl, $\Delta T_m = T_m$ (DNA + ligand) − $T_m$ (DNA). The values were obtained as the mean ± SD from at least three independent measurements.
[b] $\Delta T_m$ values for incubation of 0.2 μM F10T with and without 2.0 μM compound and 0.2 mM KCl, $\Delta T_m = T_m$ (DNA + ligand) − $T_m$ (DNA). The values were obtained as the mean ± SD from at least three independent measurements.
[c] The values were obtained with SPR as the mean ± SD from at least two independent measurements.
[d] No obvious binding even with 10 μM of compounds.

immobilized *c-myc* DNA and duplex DNA with blank reference (Fig. S1). The binding constants were determined through equilibrium analysis as shown in Table 1. The $K_D$ values of the derivatives interacting with *c-myc* G-quadruplex DNA fell into a range of 0.4–4.4 μM, which demonstrated that all the derivatives had a high binding affinity to *c-myc* G-quadruplex. Our SPR results showed that the methylated compounds had higher $K_D$ values (0.4–1.2 μM) than non-methylated ones (1.4–4.4 μM), which was consistent with the FRET results, indicating that the positive charge played a significant role in binding with *c-myc* G-quadruplex. Both FRET and SPR results showed that the pyrrolidino group of **20b** or **21b** was optimal for their interaction with *c-myc* G-quadruplex DNA, while the introduction of the cyclohexylamino group (**20e**) was unfavorable for the binding interaction. Besides, the SPR data indicated that all the derivatives had no obvious binding to duplex DNA even at a concentration of 10 μM (Table 1, Fig. S1), which was consistent with the FRET results. The results from above FRET and SPR experiments all supported that these acridine derivatives could selectively recognize *c-myc* G-quadruplex DNA over duplex DNA. The methylated derivatives had good activity in binding on and stabilizing G-quadruplex DNA, which could act as a new class of highly selective G-quadruplex binding ligands.

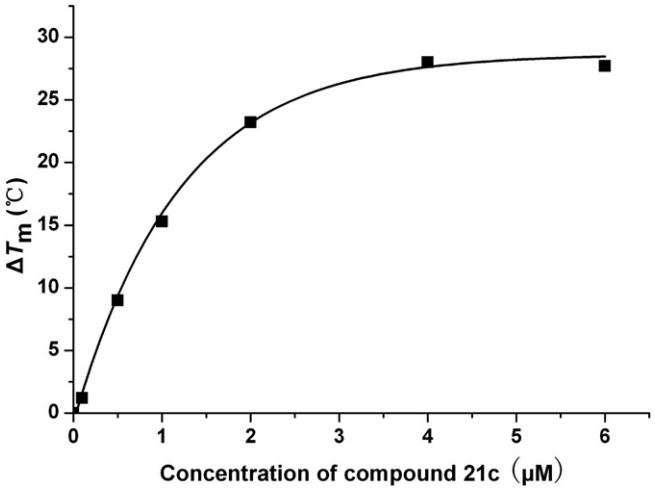



**Fig. 2.** Concentration-dependent melting curve for compound **21c** upon binding to FPu18T. The concentration of the FPu18T remained at 0.2 μM in 10 mM Tris–HCl buffer with 0.2 mM KCl, pH 7.2. The concentrations of compound **21c** were 0.1, 0.5, 1.0, 2.0, 4.0, and 6.0 μM.

### 3.3. Binding property studies with CD

The circular dichroism (CD) experiment was performed with oligonucleotide Pu27 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′ as the *c-myc* sequence according to a previous study [21]. The conformational property of *c-myc* G-quadruplex DNA treated with increasing concentrations (0.5–10 eq.) of the derivatives (**15a**, **15b**, **16a**, **17a**, **17c**, **20a**–**e**, **21b**, and **21c**) in the presence or absence of $K^+$ (100 mM) were studied by using CD spectroscopy. The CD result showed that the conformation of *c-myc* G-quadruplex had parallel structure in the presence or absence of $K^+$ (100 mM) with a positive signal at 262 nm and a negative signal at 242 nm (Fig. S2). This *c-myc* G-quadruplex structure had little change when treated with various concentrations of the derivatives either in the presence or in the absence of $K^+$ (100 mM) (Fig. S2).

### 3.4. Inhibition of amplification in the promoter region of c-myc by 5,6-dihydrobenzo[c]acridine derivatives

The inhibition of amplification of *c-myc* gene by the derivatives *in vitro* was investigated with Pu27 (5′-TGGGGAGGGTGGGGAGG GTGGGGAAGG-3′) as the *c-myc* DNA sequence by using PCR-stop

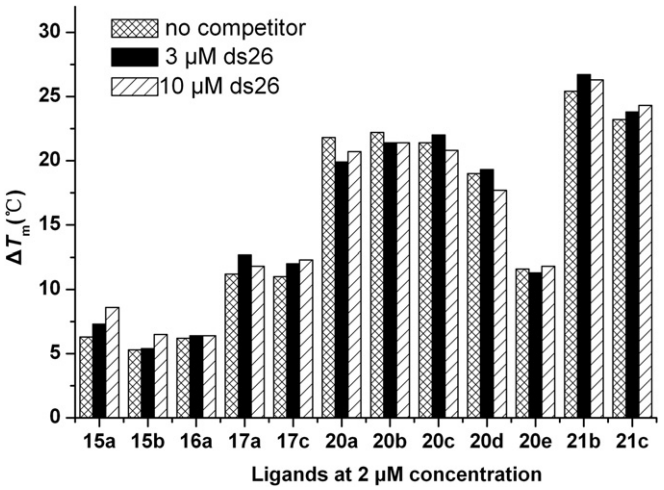



**Fig. 3.** Competitive FRET results for 5,6-dihydrobenzo[*c*]acridine derivatives without and with 3 μM or 10 μM of duplex DNA competitor (ds26). The concentration of FPu18T was 0.2 μM.

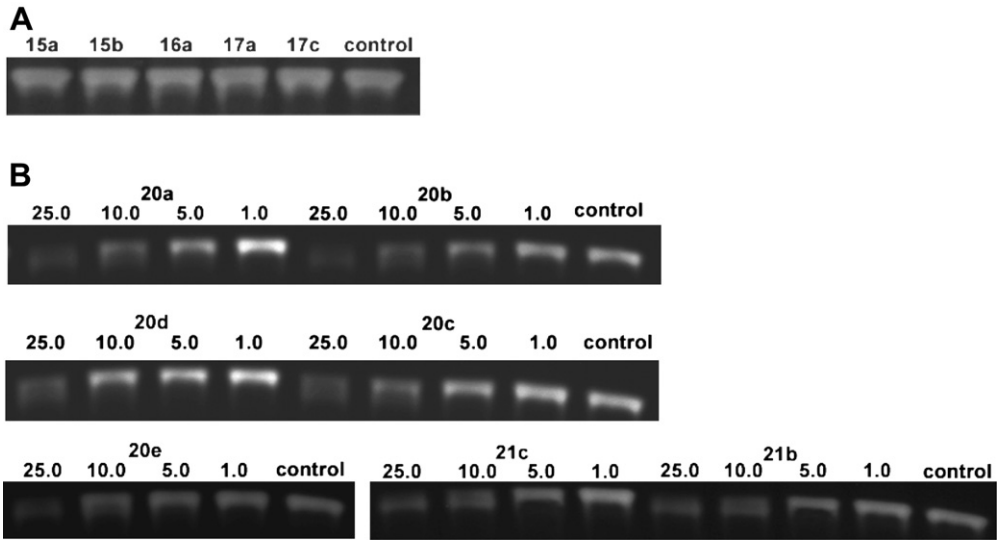



**Fig. 4.** Effect of 5,6-dihydrobenzo[*c*]acridine derivatives on the PCR-stop assay with Pu27. The white ladders were the PCR products. (A) The concentrations of compounds **15a**, **15b**, **16a**, **17a**, and **17c** were at 50 μM. (B) The increasing concentrations of compounds **20a**–**e**, **21b**, and **21c** were at 1, 5, 10, and 25 μM, respectively.

assay [21]. The compounds of various concentrations (1.0, 5.0, 10.0, and 25.0 μM) were used, and the PCR products were separated with PAGE and stained with GelRed (Fig. 4). The concentrations for 50% inhibition of amplification ($IC_{50}$) were calculated as shown in Table 2. The methylated compounds (**20a**–**e** and **21b**–**c**) showed good inhibitory effect on the hybridization of Pu27 with the $IC_{50}$ values ranged from 4.7 to 14.0 μM (Table 2), while the non-methylated ones had weak inhibitory activity ($IC_{50} > 50$ μM), which were consistent with the FRET and SPR results and confirmed the importance of the nitrogen methylation in the binding of the ligands to the *c-myc* G-quadruplex DNA.

Compared with compounds **20b** and **20c**, compounds **21b** and **21c** had higher inhibition activity, indicating the 1,3-dioxole ring indeed was not a better structural building block than the opened *o*-dimethoxyl group for the ligands to interact with G-quadruplex. It might be the result of the two methoxy groups could adjust themselves better to accommodate the G-quartet surface, while the rigid 1,3-dioxole ring in compounds **20b** and **20c** could not. The PCR result also showed that compound **20e** had lower inhibition activity, which was consistent with the FRET and SPR results, indicating the cyclohexylamino was not a suitable group in designing G-quadruplex ligands.

### 3.5. Molecular modeling studies

In order to better understand how the nearly planar crescent-shaped ligands interact with *c-myc* G-quadruplex DNA through π-orbital stacking, we carried out the modeling study for the binding of compound **21c**, **20c** and **17c** with the *c-myc* G-quadruplex (Fig. 5). The *c-myc* G-quadruplex model and the ligands were modified according to the previously reported procedure [45]. The modeling results showed that these three ligands could stack on both external G-quartet surfaces, with preference to stack on the 5′ G-quartet surface. As shown in Fig. 5, these three compounds had similar binding mode with the G-quadruplex, and their nearly planar scaffolds were stacked on the G-quartet surface through π-orbital overlapping, while their protonated side chains were oriented on the loops of the G-quadruplexes through electrostatic interactions with the phosphate backbone. The binding free energy showed that compounds **21c** ($\Delta G = -7.05$ kcal·mol$^{-1}$) and **20c** ($\Delta G = -6.75$ kcal·mol$^{-1}$) had better interactions with G-quadruplex than compound **17c** ($\Delta G = -6.38$ kcal·mol$^{-1}$). The positively charged groups of compounds **21c** and **20c** were located above the negative oxygen channel of G-quadruplex, while the weakly protonated nitrogen of compound **17c** had a certain degree of deviation from this negative channel. These results showed that the methylation of the nitrogen atom provided more effective ligand than its weak protonation in the design of new G-quadruplex binding ligands.

The above modeling study results showed that the two methoxy groups of compound **21c** could adjust their locations and orientations based on their surroundings, while the 1,3-dioxole ring of compound **20c** could only orient itself to the groove of G-quadruplex, which might result in the lowered $\Delta G$ value of **21c** (−7.05 kcal·mol$^{-1}$) compared with that of **20c** (−6.75 kcal·mol$^{-1}$). This result indicated that the two free methoxy groups were more effective for 5,6-dihydrobenzo[*c*]acridine derivatives to interact with G-quadruplex than the rigid 1,3-dioxole ring.

### 3.6. Cell proliferation assay and inhibition of c-myc DNA transcription by 5,6-dihydrobenzo[c]acridine derivatives in cancer cell line

To evaluate the inhibitory activity of the derivatives for cancer cell lines, the short-term proliferation assay of two cell lines, Ramos and CA46, treated with compound **21c** was studied. The NHE $III_1$ element of *c-myc* gene was retained in the Ramos cell line, which was removed together with the P1 and P2 promoters in the CA46 cell line [48,49]. The cells were incubated with compound **21c** of varying concentrations (2.5, 5, and 10 μM), and the experiments were carried out for four days with cell counts and viability determined every day. As shown in Fig. 6, the concentration-dependent inhibition for the proliferation of Ramos by compound **21c** was observed, while for cell line CA46 whose NHE $III_1$ element

**Table 2**
The $IC_{50}$ values of compounds in PCR-stop assay.

| Compounds | 15a | 15b | 16a | 17a | 17c | 20a | 20b | 20c | 20d | 20e | 21b | 21c |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $IC_{50}$ (μM) | >50 | >50 | >50 | >50 | >50 | 7.5 | 5.3 | 5.4 | 9.5 | 14.0 | 4.7 | 5.0 |

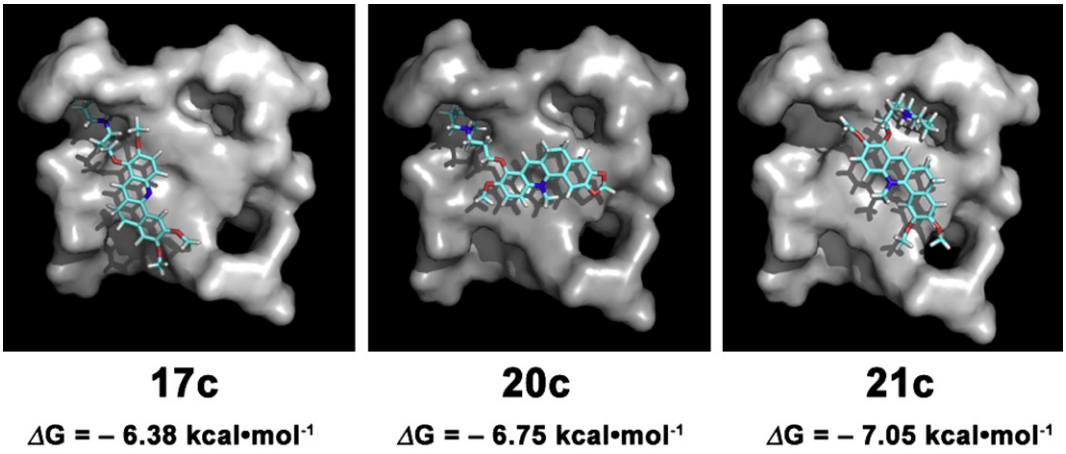



**Fig. 5.** Modeling studies for the complexes of the ligands (**17c**, **20c**, and **21c**) and G-quadruplex. Pictures were generated by using PyMOL.

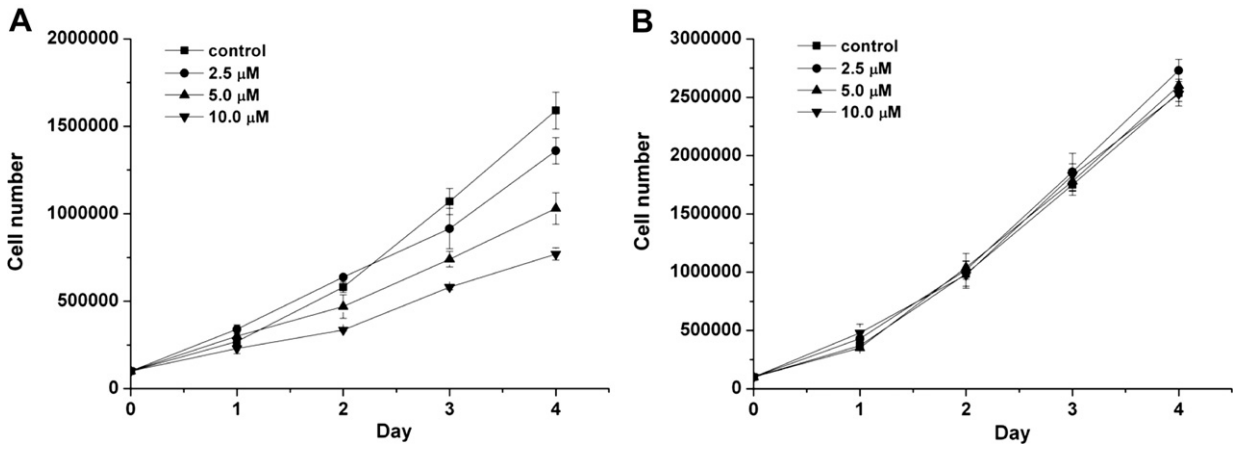



**Fig. 6.** Effect of compound **21c** on the proliferation of (A) Ramos cells and (B) CA46 cells. The cells were exposed to the indicated concentrations of compound **21c** or 0.1% DMSO (control), respectively. Every day, the cells in control and drug-exposed well were counted. Each experiment was performed three times for each point.

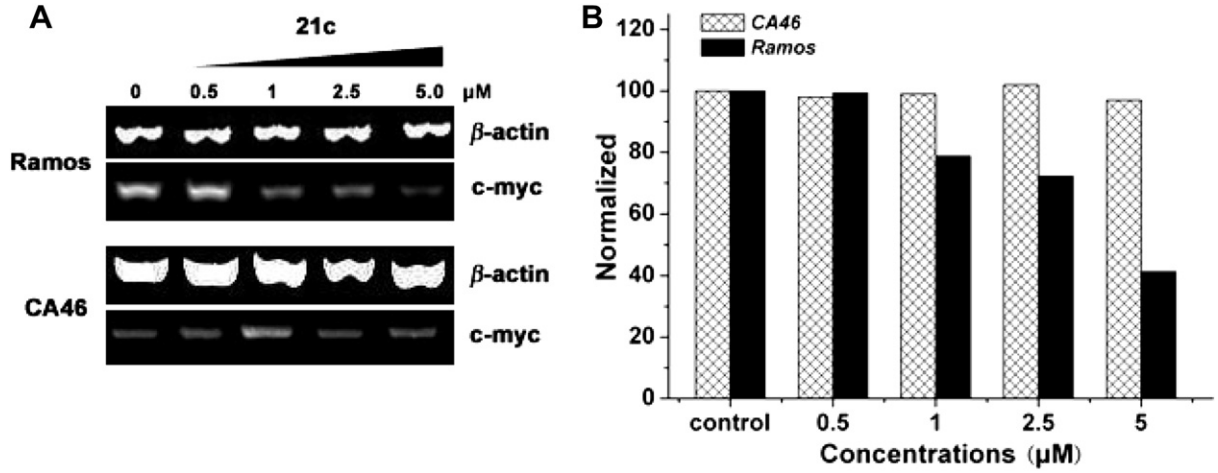



**Fig. 7.** RT-PCR assay for the effect of compound **21c**. Ramos cells and CA46 cells were treated with varying concentrations of compound **21c** (0.5, 1, 2.5, and 5 μM), and the transcriptions of *c-myc* gene were determined. *c-myc* mRNA level was normalized to β-actin mRNA level for each sample.

was removed, the cell proliferation was not obviously affected by compound **21c**.

Following the cell proliferation assay experiments, the transcriptions of *c-myc* gene in Ramos cell line and CA46 cell line were also studied. Ramos cells and CA46 cells were incubated with compound **21c** at the concentrations of 0.5, 1, 2.5, and 5 μM for 4 days. The total RNA was extracted and reversely transcripted for cDNA, which was used as a template for specific PCR amplification of the *c-myc* gene with β-actin as a control. As shown in Fig. 7, the ligand **21c** showed good concentration-dependent inhibition on the transcription of *c-myc* gene in Ramos cells, which was not observed in CA46 cell line. The above results suggested that the derivatives might target the G-quadruplex structure in the promoter region of *c-myc* gene and hence inhibited its expression.

## 4. Conclusion

The interaction between crescent-shaped 5,6-dihydrobenzo[*c*]acridine derivatives (**15a**, **15b**, **16a**, **17a**, **17c**, **20a**–**e**, **21b**, and **21c**) and G-quadruplex DNA in promoter region of *c-myc* gene was studied in detail. According to the FRET, SPR, and PCR-stop results, it could be concluded that the nitrogen atom in the 5,6-dihydrobenzo[*c*]acridine scaffold had weak tendency of protonation under physiological conditions. Besides the π-orbital stacking of the scaffold and the electrostatic interactions of the side chain, the electrostatic interactions between the positive charge at 12-*N*-position and the negative oxygen channel of G-quadruplex made an important contribution for the derivatives to strongly bind on and stabilize the *c-myc* G-quadruplex. Our results also indicated that the two methoxy groups in the 5,6-dihydrobenzo[*c*]acridine scaffold could be more suitable for the derivatives to interact with G-quadruplex than the 1,3-dioxole ring. Their significant activities of inhibiting the *c-myc* transcription in Ramos cell line but not in CA46 cell line might be due to stabilization of G-quadruplex structure by the 12-*N*-methylated 5,6-dihydrobenzo[*c*]acridine derivatives. Thus we might conclude that methylation on nitrogen atom in certain molecular scaffold which generates a positive charge, is important in designing a new G-quadruplex ligand, and the methylated derivatives with high selectivity toward G-quadruplex DNA over duplex DNA might become promising lead compounds for developing anti-cancer agents.

## 5. Experimental section

### 5.1. Synthesis and characterization

$^1$H and $^{13}$C NMR spectra were recorded using TMS as the internal standard in DMSO-$d_6$, $D_2O$ or $CDCl_3$ with a Bruker BioSpin GmbH spectrometer at 400 MHz; Mass spectra (MS) were recorded on a Shimadzu LCMS-2010A instrument with an ESI-ACPI mass selective detector, and high resolution mass spectra (HRMS) were recorded on Shimadzu LCMS-IT-TOF. All the compounds were purified by using flash column chromatography with silica gel (200–300 mesh) or $Al_2O_3$ (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. Their purities were proved to be higher than 95% by using analytical HPLC equipped with Shimadzu LC-20AB system and an Ultimate XB-C18 column (4.6 × 250 mm, 5 μm), which was eluted with methanol–water (35:65–50:50) containing 0.1% TFA at a flow rate of 0.5 mL·min$^{-1}$. Melting points (Mp) were determined using a SRS-OptiMelt automated melting point instrument without correction.

#### 5.1.1. Synthesis of 4-(3,4-dimethoxyphenyl)-4-oxobutanoic acid (**3**)

$AlCl_3$ (2.65 mol, 353.0 g) was slowly added to the mixture of 1,2-dimethoxybenzene (1.06 mol, 146.0 g) in nitrobenzene (1.5 L) at 0 °C over about 0.5 h, and then the dihydrofuran-2,5-dione (1.27 mol, 127 g) was slowly added to the above mixture over 1 h. After finishing addition, the mixture was continuously stirred for 10 min at this temperature, and then stirred at 60 °C for about 3 h. The resulting mixture was slowly added into the crashed ice, and the precipitate was filtered and dried. The crude product was recrystallized from the ethyl acetate, and compound **3** was obtained as a white solid (60 g, 24%), Mp: 161–163 °C (lit: 158–160 °C [50]). $^1$H NMR (400 MHz, $CDCl_3$): δ 10.83 (s, 1H), 7.61 (dd, $J$ = 2 Hz, $J$ = 2 Hz, 1H), 7.53 (d, $J$ = 1.6 Hz, 1H), 6.89 (d, $J$ = 8.4 Hz, 1H), 3.94 (s, 3H), 3.92(s, 3H), 3.28 (t, $J$ = 6.8 Hz, 2H), 2.79 (t, $J$ = 6.8 Hz, 2H); MS (ESI + APCI) $m/z$: 237.1 $[M - H]^-$.

#### 5.1.2. Synthesis of 4-(3,4-dimethoxyphenyl)butanoic acid (**4**)

Triethylsilane (0.78 mol, 125 mL) was added into the mixture of the compound **3** (0.19 mol, 46 g) in $CF_3CO_2H$ (140 mL), and the resulting solution was heated under reflux for 2 h. Then the solvent was removed under reduced pressure, and the residue was poured into the crashed ice. The precipitate was filtered and dried, and compound **4** was obtained as a pale solid (42 g, 97.7%), Mp: 59–61 °C (lit: 60–61 °C [50]). $^1$H NMR (400 MHz, $CDCl_3$): δ 11. 80 (s, 1H), 6.79 (d, $J$ = 7.6 Hz, 1H), 6.73 (d, $J$ = 1.6 Hz, 1H), 6.71 (s, 1H), 3.87 (s, 3H), 3.85 (3, 3H), 2.62 (t, $J$ = 7.6 Hz, 2H), 2.38 (t, $J$ = 7.6 Hz, 2H), 1.99–1.91 (m, 2H); MS (ESI + APCI) $m/z$: 223.1 $[M - H]^-$.

#### 5.1.3. Synthesis of 6,7-dimethoxy-3,4-dihydronaphthalen-1(2H)-one (**5**)

Polyphosphoric acid (210 g) was added into the compound **4** (0.19 mol, 42 g) dissolved in dichloromethane (50 mL), and the mixture was heated under reflux for 2 h. After the reaction was finished, the solvent was removed and the residue was slowly added into the crashed ice. The pH value of the solution was adjusted to 7–8, and the precipitate was filtered and dried, and compound **5** was obtained as a white solid (36 g, 93.3%), Mp: 94–96 °C (lit: 96–98 °C [50]). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.51 (s, 1H), 6.67 (s, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 2.89 (t, $J$ = 6 Hz, 2H), 2.60 (t, $J$ = 6.4 Hz, 2H), 2.15–2.09 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 197.10, 153.51, 147.96, 139.27, 125.79, 110.22, 108.57, 55.98, 38.50, 29.44, 23.61.

#### 5.1.4. Synthesis of 6,7-dihydroxy-3,4-dihydronaphthalen-1(2H)-one (**6**)

The compound **5** (0.19 mol, 34 g) was added into 48% HBr (500 mL), and the mixture was heated under reflux for 4 h, and then the mixture was cooled to room temperature. The brown crystal was filtered and dried, and compound **6** was obtained (24.6 g, 84.8%), Mp: 198–200 °C. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.67 (s, 1H), 7.16 (s, 1H), 6.75 (s, 1H), 6.23 (s, 1H), 2.85 (t, $J$ = 6.4 Hz, 2H), 2.59 (t, $J$ = 6.4 Hz, 2H), 2.12–2.06 (m, 2H); MS (ESI + APCI) $m/z$: 177.1 $[M - H]^-$.

#### 5.1.5. Synthesis of 7,8-dihydronaphtho[2,3-d][1,3]dioxol-5(6H)-one (**7**)

To a reaction flask, compound **6** (0.14 mol, 24.6 g), KF (1.24 mol, 72 g), dibromomethane (0.17 mol, 11.6 mL) and DMF (300 mL) were added, and the mixture was heated at 140 °C for 6 h. After the reaction was cooled to room temperature, the solvent was removed and the residue was extracted with ethyl acetate. The solution was washed with water, dried, and purified with gel chromatography to give compound **7** as a white solid (14.2 g, 54%), Mp: 75–78 °C (lit: 75 °C [51]). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.45 (s, 1H), 6.65 (s, 1H), 5.99 (s, 2H), 2.86 (t, $J$ = 6 Hz, 2H), 2.58 (t, $J$ = 6.4 Hz, 2H), 2.11–2.05 (m, 2H), $^{13}$C NMR (101 MHz, $CDCl_3$): δ 196.53, 151.99, 146.91, 141.35, 127.47, 107.91, 106.23, 101.52, 38.61, 30.02, 23.46.

#### 5.1.6. Synthesis of the intermediate 6-amino-3-methoxy-2-(phenylsulfonyloxy)benzoic acid (**9**)

$KMnO_4$ (0.33 mol, 53 g) dissolved in water (100 mL) was slowly added into a solution containing compound **8** (0.22 mol, 76 g) [40]

and acetone (300 mL) at room temperature, and the resulting mixture was stirred for about 2 h. After the reaction was completed, isopropyl alcohol (20 mL) was added, and the mixture was continuously stirred for another 1 h. The mixture was filtered on celite, and the solvent was removed under reduced pressure. The resulting white solid was directly used for the next step.

The obtained white solid was added into acetic acid/ethanol/water (2:2:1, 500 mL), and Fe powder (3.4 mol, 189 g) was added in portion. The mixture was stirred at room temperature for about 4 h, and white solid appeared and was filtered. The solid was dissolved in 5 M NaOH (100 mL), and the resulting solution was filtered on celite. After the pH value of the filtrate was adjusted to 5–6, the precipitate appeared. The crude product was filtered and dried to give compound **9** as a pale solid (30 g, 41.7%), Mp: 150–153 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.89 (d, $J$ = 7.6 Hz, 2H), 7.65 (t, $J$ = 7.6 Hz, 1H), 7.52 (t, $J$ = 7.6 Hz, 2H), 6.92 (d, $J$ = 8.8 Hz, 1H), 6.59 (d, $J$ = 9.2 Hz, 1H), 3.38 (s, 3H), $^{13}$C NMR (101 MHz, DMSO-$d_6$): $\delta$ 167.31, 143.96, 142.16, 136.34, 136.26, 134.35, 129.21, 128.10, 118.54, 115.50, 110.79, 56.07; MS (ESI + APCI) $m/z$: 324 $[M + H]^+$.

### 5.1.7. General procedure for preparation of compounds **10** and **11**

The mixture of compound **7** or **5** (1 eq.) and compound **9** (1.5 eq.) was heated under reflux in phosphorus oxychloride for 8 h. The mixture was cooled down to room temperature, and slowly poured into the crashed ice. The precipitate was filtered, dissolved in dichloromethane, washed with saturated $NaHCO_3$, dried, and purified by using flash column chromatography to give compound **10** or **11** as a light yellow solid.

#### 5.1.7.1. 7-Chloro-9-methoxy-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-8-yl benzenesulfonate (**10**).

Following the general procedure, compound **7** (0.075 mol, 14.2 g) and compound **9** (0.11 mol, 36 g) were used, and the desired product was obtained as a light yellow solid (23.7 g, 64%), Mp: 201–204 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.08 (d, $J$ = 8.0 Hz, 1H), 8.00 (s, 1H), 7.87 (d, $J$ = 7.6 Hz, 2H), 7.66 (t, $J$ = 7.6 Hz, 1H), 7.53 (t, $J$ = 8.0 Hz, 2H), 7.32 (d, $J$ = 9.2 Hz, 1H), 6.73 (s, 1H), 6.01 (s, 2H), 3.43 (s, 3H), 3.24 (t, $J$ = 7.6 Hz, 2H), 2.91 (t, $J$ = 7.6 Hz, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 151.39, 150.35, 149.35, 147.31, 143.15, 137.83, 135.78, 134.19, 133.57, 130.87, 130.76, 130.12, 128.61, 128.49, 127.63, 121.50, 115.86, 107.78, 106.15, 101.25, 55.91, 27.48, 26.02; MS (ESI + APCI) $m/z$: 496.0 $[M + H]^+$.

#### 5.1.7.2. 7-Chloro-2,3,9-trimethoxy-5,6-dihydrobenzo[c]acridin-8-yl benzenesulfonate (**11**).

Following the general procedure, compound **5** (9.7 mmol, 2 g) and compound **9** (15.5 mmol, 5 g) were used, and the desired product was obtained as a light yellow solid (2.4 g, 48.4%), Mp: 210–211 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.20 (s, 1H), 8.09 (s, 1H), 7.88 (d, $J$ = 0.8 Hz, 1H), 7.86 (d, $J$ = 1.6 Hz, 1H), 7.66 (t, $J$ = 7.6 Hz, 1H), 7.53 (t, $J$ = 8 Hz, 2H), 7.33 (d, $J$ = 9.2 Hz, 1H), 6.76 (s, 1H), 4.06 (s, 3H), 3.96 (s, 3H), 3.43 (s, 3H), 3.27 (t, $J$ = 7.6 Hz, 2H), 2.95 (t, $J$ = 7.6 Hz, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 151.51, 151.03, 150.38, 148.51, 143.17, 137.86, 135.89, 133.55, 132.57, 130.99, 130.80, 129.99, 128.60, 128.49, 126.06, 121.49, 115.89, 110.42, 108.73, 56.18, 55.98, 55.96, 27.01, 26.08; MS (ESI + APCI) $m/z$: 512.1 $[M + H]^+$.

### 5.1.8. General procedure for preparation of compounds **12**–**14**

Compound **10** or **11** was dechlorinated under $H_2$ and 10% Pd/C (1 eq. wt %) at atmospheric pressure in DMF/ethanol at 60 °C for 3 h. The mixture was filtered through celite, and the solvent was removed. The residue was dissolved in dioxane, and 5 M NaOH (5 eq.) was added, and the mixture was kept at 100 °C for 2 h. Then dioxane was removed, and the pH value of the solution was adjusted to 6–7. A light red solid appeared, which was filtered and dried. Then the solid was dissolved in acetonitrile, and dibromoalkanes (5 eq.) and $K_2CO_3$ (3 eq.) were added. The mixture was kept at 60 °C for 3 h, and the solvent was removed, and the residue was purified by using flash column chromatography to afford compound **12**, **13** or **14** as a light yellow solid.

#### 5.1.8.1. 8-(2-Bromoethoxy)-9-methoxy-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]-acridine (**12**).

Following the general procedure, compound **10** (2 mmol, 1.0 g) and 1,2-dibromoethane (10.6 mmol, 2.0 g) were used, and the desired product was obtained as a light yellow solid (0.26 g, 30%), Mp: 158–160 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.31 (s, 1H), 8.02 (s, 1H), 7.87 (d, $J$ = 9.2 Hz, 1H), 7.41 (d, $J$ = 9.2 Hz, 1H), 6.73 (s, 1H), 6.00 (s, 2H), 4.50 (t, $J$ = 6.0 Hz, 2H), 4.00 (s, 3H), 3.73 (t, $J$ = 6.0 Hz, 2H), 3.11 (t, $J$ = 7.6 Hz, 2H), 2.91 (t, $J$ = 7.6 Hz, 2H); MS (ESI + APCI) $m/z$: 428.0 (93.5%), 430.1 (100%) $[M + H]^+$.

#### 5.1.8.2. 8-(3-Bromopropoxy)-9-methoxy-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]-acridine (**13**).

Following the general procedure, compound **10** (4 mmol, 2.0 g) and 1,3-dibromopropane (20 mmol, 3.8 g) were used, and the desired product was obtained as a light yellow solid (0.62 g, 35%), Mp: 132–135 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.74 (s, 1H), 8.55 (d, $J$ = 9.6 Hz, 1H), 8.09 (s, 1H), 7.71 (d, $J$ = 9.2 Hz, 1H), 6.83 (s, 1H), 6.14 (s, 2H), 4.36 (t, $J$ = 5.6, 2H), 4.05 (s, 3H), 3.78 (t, $J$ = 5.6 Hz, 2H), 3.19 (t, $J$ = 7.6 Hz, 2H), 3.00 (t, $J$ = 7.6 Hz, 2H), 2.45–2.39 (m, 2H); MS (ESI + APCI) $m/z$: 442.1 (75.5%), 444.1 (100%) $[M + H]^+$.

#### 5.1.8.3. 8-(3-Bromopropoxy)-2,3,9-trimethoxy-5,6-dihydrobenzo[c]acridine (**14**).

Following the general procedure, compound **11** (4.7 mmol, 2.4 g) and 1,3-dibromopropane (25 mmol, 4.7 g) were used, and the desired product was obtained as a light yellow solid (1.24 g, 57.6%), Mp: 126–127 °C. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.17 (s, 1H), 8.08 (s, 1H), 7.89 (d, $J$ = 8.8 Hz, 1H), 7.41 (d, $J$ = 9.2 Hz, 1H), 6.76 (s, 1H), 4.28 (t, $J$ = 6.0 Hz, 2H), 4.06 (s, 3H), 4.00 (s, 3H), 3.95 (s, 3H), 3.78 (t, $J$ = 6.4 Hz, 2H), 3.13 (t, $J$ = 7.6 Hz, 2H), 2.95 (t, $J$ = 7.6 Hz, 2H), 2.45–2.39 (m, 2H); MS (ESI + APCI) $m/z$: 458.1 (100%), 460.1 (94%) $[M + H]^+$.

### 5.1.9. General procedure for preparing of compound **15a**, **15b**, **16a**, **17a**, **17c**

The compound **12**, **13** or **14** (1 eq.) was dissolved in acetonitrile (10–100 mL), and the alkyl amine (1.5–10 eq.) and $K_2CO_3$ (3 eq.) were added, and the mixture was stirred at 60 °C for about 3–6 h. The solvent was removed under reduced pressure, and the residue was washed with water, extracted with dichloromethane, dried and purified by using flash column chromatography to give the desired compound **15a**, **15b**, **16a** or **17a**, **17c** as a pale solid.

#### 5.1.9.1. N,N-Diethyl-2-((9-methoxy-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-8-yl)oxy)ethanamine (**15a**).

Following the general procedure, compound **12** (0.14 mmol, 60 mg) and diethylamine (1.4 mmol, 0.144 mL) were used, and the desired product was obtained as a pale solid (17 mg, 29%). Purity: 98.4% (by HPLC, MeOH/$H_2O$). Mp: 90–91 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.26 (s, 1H), 8.01(s, 1H), 7.83 (d, $J$ = 9.2 Hz, 1H), 7.40 (d, $J$ = 9.2 Hz, 1H), 6.73 (s, 1H), 6.00 (s, 2H), 4.25 (t, $J$ = 6.0 Hz, 2H), 3.99 (s, 3H), 3.10–3.07 (m, 2H), 3.00 (s, 2H), 2.92–2.89 (m, 2H), 2.73 (s, 4H), 1.11 (t, $J$ = 6.8 Hz, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 151.61, 148.66, 148.02, 147.19, 143.37, 140.82, 134.21, 129.94, 128.97, 127.67, 125.21, 123.41, 116.84, 107.97, 105.95, 101.07, 71.49, 56.74, 52.84, 47.44, 29.26, 28.55, 11.72; HRMS (ESI) $m/z$: calcd for $C_{25}H_{28}N_2O_4$ $[M + H]^+$ 421.2127, found 421.2122.

#### 5.1.9.2. 9-Methoxy-8-(2-(pyrrolidin-1-yl)ethoxy)-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridine (**15b**).

Following the general procedure, compound **12** (0.14 mmol, 60 mg) and pyrrolidine (0.2 mmol, 0.017 mL) were used, and the desired product was

obtained as a pale solid (28 mg, 46.7%). Purity: 100% (by HPLC, MeOH/$H_2O$). Mp: 118–120 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.20 (s, 1H), 7.95 (s, 1H), 7.78 (d, $J$ = 9.2 Hz, 1H), 7.34 (d, $J$ = 9.2 Hz, 1H), 6.67 (s, 1H), 5.94 (s, 2H), 4.21 (t, $J$ = 5.6 Hz, 2H), 3.93 (s, 3H), 3.04–3.00 (m, 2H), 2.92 (s, 2H), 2.86–2.82 (m, 2H), 2.62 (s, 4H), 1.80 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 151.62, 148.70, 148.18, 147.24, 143.48, 140.96, 134.21, 129.92, 129.06, 127.72, 125.30, 123.58, 117.04, 107.97, 106.01, 101.07, 72.09, 56.85, 55.95, 54.52, 29.30, 28.61, 23.61; HRMS (ESI) $m/z$: calcd for $C_{25}H_{26}N_2O_4$ $[M + H]^+$ 419.1971, found 419.1969.

*5.1.9.3. N,N-Diethyl-3-((9-methoxy-5,6-dihydro-[1,3]dioxolo [4′,5′:4,5]benzo[1,2-c]acridin-8-yl)oxy)propan-1-amine (**16a**).* Following the general procedure, compound **13** (0.59 mmol, 260 mg) and diethylamine (5.9 mmol, 0.61 mL) were used, and the desired product was obtained as a pale solid (230 mg, 90.2%). Purity: 100% (by HPLC, MeOH/$H_2O$). Mp: 91–93 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.16 (s, 1H), 8.01 (s, 1H), 7.83 (d, $J$ = 9.2 Hz, 1H), 7.40 (d, $J$ = 9.2 Hz, 1H), 6.72 (s, 1H), 5.99 (s, 2H), 4.19 (t, $J$ = 6.4 Hz, 2H), 3.98 (s, 3H), 3.10–3.07 (m, 2H), 2.92–2.88 (m, 2H), 2.85–2.81 (m, 2H), 2.67 (q, $J$ = 7.2 Hz, 4H), 2.12–2.05 (m, 2H), 1.11 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 151.59, 148.69, 148.04, 147.22, 143.51, 140.98, 134.18, 129.90, 128.99, 127.42, 125.16, 123.41, 117.13, 107.95, 105.98, 101.06, 72.18, 56.84, 49.79, 46.92, 29.28, 28.56, 27.71, 11.41; HRMS (ESI) $m/z$: calcd for $C_{26}H_{30}N_2O_4$ $[M + H]^+$ 435.2284, found 435.2279.

*5.1.9.4. N,N-Diethyl-3-((2,3,9-trimethoxy-5,6-dihydrobenzo[c]acridin-8-yl)oxy)propan-1-amine (**17a**).* Following the general procedure, compound **14** (0.13 mmol, 60 mg) and diethylamine (0.2 mmol, 0.02 mL) were used, and the desired product was obtained as a pale solid (20 mg, 34.2%). Purity: 99.7% (by HPLC, MeOH/$H_2O$). Mp: 95–98 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.14 (s, 1H), 8.06 (s, 1H), 7.86 (d, $J$ = 9.2 Hz, 1H), 7.40 (d, $J$ = 9.2 Hz, 1H), 6.75 (s, 1H), 4.20 (t, $J$ = 6.0 Hz, 2H), 4.05 (s, 3H), 3.98 (s, 3H), 3.94 (s, 3H), 3.13–3.10 (m, 2H), 2.99–2.92 (m, 4H), 2.82–2.77 (m, 4H), 2.16 (t, $J$ = 7.6 Hz, 2H), 1.19 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 151.73, 150.30, 148.39, 147.93, 143.41, 140.67, 132.64, 130.14, 127.37, 127.35, 125.22, 123.26, 116.79, 110.63, 108.34, 71.70, 56.73, 56.11, 55.93, 49.66, 46.82, 29.37, 28.03, 26.95, 10.67; HRMS (ESI) $m/z$: calcd for $C_{27}H_{34}N_2O_4$ $[M + H]^+$ 451.2591, found 451.2596.

*5.1.9.5. 2,3,9-Trimethoxy-8-(3-(piperidin-1-yl)propoxy)-5,6-dihydrobenzo[c]acridine (**17c**).* Following the general procedure, compound **14** (0.13 mmol, 60 mg) and piperidine (0.2 mmol, 0.02 mL) were used, and the desired product was obtained as a pale solid (24 mg, 40%). Purity: 99.9% (by HPLC, MeOH/$H_2O$). Mp: 128–129 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.15 (s, 1H), 8.08 (s, 1H), 7.86 (d, $J$ = 9.2 Hz, 1H), 7.40 (d, $J$ = 9.2 Hz, 1H), 6.75 (s, 1H), 4.20 (t, $J$ = 6.4 Hz, 2H), 4.06 (s, 3H), 3.99 (s, 2H), 3.95 (s, 3H), 3.12 (t, $J$ = 7.2 Hz, 2H), 2.96–2.92 (m, 2H), 2.67 (s, 2H), 2.49 (s, 4H), 2.13 (s, 2H), 1.66 (s, 4H), 1.48 (s, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 151.71, 150.33, 148.45, 148.05, 143.54, 140.96, 132.58, 130.03, 127.52, 127.49, 125.16, 123.42, 117.05, 110.70, 108.46, 72.06, 56.86, 56.15, 56.04, 55.97, 54.21, 29.41, 28.09, 27.49, 25.58, 24.18; HRMS (ESI) $m/z$: calcd for $C_{28}H_{34}N_2O_4$ $[M + H]^+$ 463.2597, found 463.2588.

*5.1.10. General procedure for preparation of compounds **18**, **19***

To a stirred solution of compound **13** or **14** (1 eq.) in toluene, methyl triflate (1.2–2.0 eq.) was slowly added at room temperature, and a light yellow solid appeared in 5 min. After the reaction was continuously stirred for about 1 h, the solid was filtered and dried to afford the desired compound **18** or **19** as a yellow solid.

*5.1.10.1. 8-(3-Bromopropoxy)-9-methoxy-12-methyl-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium trifluoromethanesulfonate (**18**).* Following the general procedure, compound **13** (1.13 mmol, 500 mg) and methyl triflate (1.3 mmol, 0.15 mL) were used, and the desired product was obtained as a yellow solid (437 mg, 64%), Mp: 194–197 °C. $^1$H NMR (400 MHz, $CDCl_3$): δ 8.80 (s, 1H), 8.28 (d, $J$ = 9.2 Hz, 1H), 7.86 (d, $J$ = 9.2 Hz, 1H), 7.43 (s, 1H), 6.94 (s, 1H), 6.15 (s, 2H), 4.73 (s, 3H), 4.38 (t, $J$ = 5.6 Hz, 2H), 4.08 (s, 3H), 3.76 (t, $J$ = 6.4 Hz, 2H), 3.10 (t, $J$ = 7.6 Hz, 2H), 2.96 (t, $J$ = 7.6 Hz, 2H), 2.46–2.40 (m, 2H); MS (ESI + APCI) $m/z$: 456.1 (100%), 458.1 (99.5%) $[M - OTf_3]^+$.

*5.1.10.2. 8-(3-Bromopropoxy)-2,3,9-trimethoxy-12-methyl-5,6-dihydrobenzo[c]acridin-12-ium trifluoromethanesulfonate (**19**).* Following the general procedure, compound **14** (0.92 mmol, 420 mg) and methyl triflate (1.9 mmol, 0.21 mL) were used, and the desired product was obtained as a yellow solid (283 mg, 50%), Mp: 186–187 °C. $^1$H NMR (400 MHz, $CDCl_3$): δ 8.77 (s, 1H), 8.27 (d, $J$ = 9.6 Hz, 1H), 7.82 (d, $J$ = 10.0 Hz, 1H), 7.57 (s, 1H), 6.93 (s, 1H), 4.79 (s, 3H), 4.37 (t, $J$ = 6.0 Hz, 1H), 4.07 (s, 3H), 4.03 (s, 6H), 3.76 (t, $J$ = 6.4 Hz, 2H), 3.10 (t, $J$ = 7.2 Hz, 2H), 2.96 (t, $J$ = 7.2 Hz, 2H), 2.45–2.40 (m, 2H); MS (ESI + APCI) $m/z$: 472.1 (90%), 474.1 (100%) $[M - OTf_3]^+$.

*5.1.11. General procedure for preparation of compounds **20a–e**, **21b**, **21c***

Compound **18** or **19** (1 eq.) was dissolved in dichloromethane, and amine (1.2–10 eq.) and $K_2CO_3$ (3 eq.) with *cat.* KI were added. The reaction mixture was stirred for 2 days at room temperature, and then washed with water and saturated NaCl, dried and purified by using flash column chromatography. The anion ($OTf_3^-$) was exchanged into chloride form ($Cl^-$) with ion-exchange resin, and the obtained residue was purified again by using $Al_2O_3$ chromatography to give the desired compound as a light yellow solid.

*5.1.11.1. 8-(3-(Diethylamino)propoxy)-9-methoxy-12-methyl-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium chloride (**20a**).* Following the general procedure, compound **18** (0.5 mmol, 300 mg) and diethylamine (1.3 mmol, 0.13 mL) were used, and the desired product was obtained as a yellow solid (60 mg, 25%). Purity: 99.5% (by HPLC, MeOH/$H_2O$). Mp: 149–150 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.79 (s, 1H), 8.71 (d, $J$ = 9.6 Hz, 1H), 7.92 (d, $J$ = 9.6 Hz, 1H), 7.66 (s, 1H), 6.92 (s, 1H), 6.14 (s, 2H), 4.96 (s, 3H), 4.27 (t, $J$ = 6.4 Hz, 2H), 4.04 (s, 3H), 3.07–3.04 (m, 2H), 2.94–2.91 (m, 2H), 2.71–2.68 (m, 2H), 2.59 (q, $J$ = 7.2 Hz, 4H), 2.05–2.01 (m, 2H), 1.05 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 153.12, 152.54, 150.46, 147.66, 141.43, 141.01, 135.31, 135.14, 133.59, 123.64, 121.94, 119.64, 116.92, 111.13, 109.00, 102.71, 73.00, 56.74, 49.36, 46.81, 46.75, 29.11, 28.83, 27.88, 11.55; HRMS (ESI) $m/z$: calcd for $C_{27}H_{33}N_2O_4$ $[M - Cl]^+$ 449.2440, found 449.2432.

*5.1.11.2. 9-Methoxy-12-methyl-8-(3-(pyrrolidin-1-yl)propoxy)-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium chloride (**20b**).* Following the general procedure, compound **18** (0.165 mmol, 100 mg) and pyrrolidine (0.2 mmol, 0.017 mL) were used, and the desired product was obtained as a yellow solid (30 mg, 37.5%). Purity: 95.4% (by HPLC, MeOH/$H_2O$). Mp: 125–126 °C; $^1$H NMR (400 MHz, $CDCl_3$): δ 8.83 (s, 1H), 8.30 (d, $J$ = 9.6 Hz, 1H), 7.85 (d, $J$ = 9.6 Hz, 1H), 7.45 (s, 1H), 6.94 (s, 1H), 6.15 (s, 2H), 4.75 (s, 3H), 4.33 (t, $J$ = 6.4 Hz, 2H), 4.06 (s, 3H), 3.07 (t, $J$ = 7.2 Hz, 2H), 2.96–2.93 (m, 2H), 2.78 (t, $J$ = 7.2 Hz, 2H), 2.62 (s, 4H), 2.17–2.09 (m, 2H), 1.83 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 153.17, 152.56, 150.48, 147.67, 141.43, 141.09, 135.49, 135.13, 133.71, 123.69, 121.87, 119.66, 116.80, 111.11, 109.02, 102.73, 72.60, 56.76, 54.17, 52.75, 46.64, 29.45, 29.08, 28.84, 23.46; HRMS (ESI) $m/z$: calcd for $C_{27}H_{31}N_2O_4$ $[M - Cl]^+$ 447.2284, found 447.2275.

*5.1.11.3. 9-Methoxy-12-methyl-8-(3-(piperidin-1-yl)propoxy)-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium chloride (**20c**).* Following the general procedure, compound **18** (0.5 mmol,

300 mg) and piperidine (0.7 mmol, 0.07 mL) were used, and the desired product was obtained as a yellow solid (45 mg, 18.4%). Purity: 98.8% (by HPLC, MeOH/$H_2O$). Mp: 140–142 °C; $^1$H NMR (400 MHz, $D_2O$): $\delta$ 8.41 (s, 1H), 7.85 (d, $J$ = 9.6 Hz, 1H), 7.70 (d, $J$ = 10 Hz, 1H), 7.21 (s, 1H), 6.97 (s, 1H), 6.04 (s, 2H), 4.39 (s, 3H), 3.97 (t, $J$ = 6.4 Hz, 2H), 3.91 (s, 3H), 2.89 (t, $J$ = 7.6 Hz, 2H), 2.78 (t, $J$ = 7.6 Hz, 2H), 2.41–2.33 (m, 6H), 1.86–1.79 (m, 2H), 1.48–1.45 (m, 4H), 1.35 (s, 2H); $^{13}$C NMR (101 MHz, $D_2O$): $\delta$ 153.81, 151.99, 150.02, 146.75, 142.44, 140.56, 134.87, 134.56, 134.47, 123.20, 120.85, 119.49, 115.14, 110.11, 109.09, 102.73, 73.45, 56.41, 55.12, 53.61, 44.71, 28.45, 27.94, 26.17, 24.67, 23.30; HRMS (ESI) $m/z$: calcd for $C_{28}H_{33}N_2O_4$ $[M - Cl]^+$ 461.2440, found 461.2432.

*5.1.11.4. 9-Methoxy-12-methyl-8-(3-(4-methylpiperazin-1-yl)propoxy)-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium chloride (**20d**).* Following the general procedure, compound **18** (0.5 mmol, 300 mg) and *N*-methyl piperazine (0.63 mmol, 0.07 mL) were used, and the desired product was obtained as a yellow solid (40 mg, 16%). Purity: 98.9% (by HPLC, MeOH/$H_2O$). Mp: 137–138 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.75 (s, 1H), 8.64 (d, $J$ = 9.6 Hz, 1H), 7.91 (d, $J$ = 9.6 Hz, 1H), 7.65 (s, 1H), 6.93 (s, 1H), 6.14 (s, 2H), 4.93 (s, 3H), 4.28 (t, $J$ = 6.4 Hz, 2H), 4.05 (s, 3H), 3.08–3.05 (m, 2H), 2.95–2.92 (m, 2H), 2.65–2.63 (m, 2H), 2.55 (s, 4H), 2.32 (s, 3H), 2.09–2.04 (m, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 153.23, 152.58, 150.44, 147.69, 141.34, 141.07, 135.18, 135.15, 133.67, 123.64, 121.88, 119.66, 116.80, 111.14, 109.02, 102.73, 72.64, 56.75, 54.85, 54.82, 52.95, 46.60, 45.82, 29.18, 28.83, 27.55; HRMS (ESI) $m/z$: calcd for $C_{28}H_{34}N_3O_4$ $[M - Cl]^+$ 476.2549, found 476.2541.

*5.1.11.5. 8-(3-(Cyclohexylamino)propoxy)-9-methoxy-12-methyl-5,6-dihydro-[1,3]dioxolo[4′,5′:4,5]benzo[1,2-c]acridin-12-ium chloride (**20e**).* Following the general procedure, compound **18** (0.5 mmol, 300 mg) and cyclohexylamine (5 mmol, 0.57 mL) were used, and the desired product was obtained as a yellow solid (130 mg, 51.6%). Purity: 96.1% (by HPLC, MeOH/$H_2O$). Mp: 127–128 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.79 (s, 1H), 8.69 (d, $J$ = 9.6 Hz, 1H), 7.92 (d, $J$ = 10 Hz, 1H), 7.65 (s, 1H), 6.92 (s, 1H), 6.14 (s, 2H), 4.94 (s, 3H), 4.30 (t, $J$ = 6.4 Hz, 2H), 4.05 (s, 3H), 3.08–3.05 (m, 2H), 2.94–2.89 (m, 4H), 2.50–2.45 (m, 1H), 2.08–2.05 (m, 2H), 1.94–1.91 (m, 2H), 1.75–1.71 (m, 2H), 1.27–1.20 (m, 4H), 1.13–1.06 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 153.17, 152.51, 150.40, 147.60, 141.37, 141.18, 135.48, 135.05, 133.81, 123.63, 121.81, 119.59, 116.75, 111.03, 109.03, 102.70, 72.76, 56.98, 56.75, 46.61, 43.36, 32.92, 30.44, 29.03, 28.80, 25.92, 24.98; HRMS (ESI) $m/z$: calcd for $C_{29}H_{35}N_2O_4$ $[M - Cl]^+$ 475.2597, found 475.2589.

*5.1.11.6. 2,3,9-Trimethoxy-12-methyl-8-(3-(pyrrolidin-1-yl)propoxy)-5,6-dihydrobenzo[c]acridin-12-ium chloride (**21b**).* Following the general procedure, compound **19** (0.16 mmol, 100 mg) and pyrrolidine (0.32 mmol, 0.03 mL) were used, and the desired product was obtained as a yellow solid (21 mg, 26.3%). Purity: 98.1% (by HPLC, MeOH/$H_2O$). Mp: 156–159 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.79 (s, 1H), 8.22 (d, $J$ = 9.6 Hz, 1H), 7.80 (d, $J$ = 9.6 Hz, 1H), 7.52 (s, 1H), 6.93 (s, 1H), 4.76 (s, 3H), 4.31 (t, $J$ = 6.4 Hz, 2H), 4.04 (s, 3H), 4.01 (s, 6H), 3.09–3.06 (m, 2H), 2.97–2.93 (m, 2H), 2.76 (t, $J$ = 7.6 Hz, 2H), 2.61 (s, 4H), 2.11 (d, $J$ = 6.8 Hz, 2H), 1.82 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 154.01, 153.70, 150.37, 148.53, 141.69, 139.10, 135.41, 135.12, 133.92, 123.72, 121.37, 118.59, 115.68, 113.83, 111.05, 72.77, 57.02, 56.71, 56.37, 54.21, 52.79, 45.57, 29.53, 29.12, 28.25, 23.46; HRMS (ESI) $m/z$: calcd for $C_{28}H_{35}N_2O_4$ $[M - Cl]^+$ 463.2591, found 463.2590.

*5.1.11.7. 2,3,9-Trimethoxy-12-methyl-8-(3-(piperidin-1-yl)propoxy)-5,6-dihydrobenzo[c]acridin-12-ium chloride (**21c**).* Following the general procedure, compound **19** (0.27 mmol, 170 mg) and piperidine (1.1 mmol, 0.11 mL) were used, and the desired product was obtained as a yellow solid (65 mg, 44.3%). Purity: 99.9% (by HPLC, MeOH/$H_2O$). Mp: 170–172 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.71 (s, 1H), 8.54 (d, $J$ = 9.6 Hz, 1H), 8.01 (s, 1H), 7.83 (d, $J$ = 9.6 Hz, 1H), 6.90 (s, 1H), 5.12 (s, 3H), 4.27 (t, $J$ = 6.4 Hz, 2H), 4.14 (s, 3H), 4.04 (s, 3H), 4.02 (s, 3H), 3.08–3.05 (m, 2H), 2.96–2.93 (m, 2H), 2.57 (t, $J$ = 7.6 Hz, 2H), 2.44 (s, 4H), 2.08–2.04 (m, 2H), 1.62–1.58 (m, 4H), 1.48–1.43 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$): $\delta$ 153.98, 153.64, 150.33, 148.75, 141.55, 138.39, 135.42, 134.83, 133.78, 123.71, 121.63, 118.89, 116.72, 114.99, 110.81, 72.90, 57.93, 56.74, 56.32, 55.63, 54.66, 46.73, 29.20, 28.37, 27.71, 25.93, 24.34; HRMS (ESI) $m/z$: calcd for $C_{29}H_{37}N_2O_4$ $[M - Cl]^+$ 477.2753, found 477.2743.

### 5.2. Materials

All oligomers/primers used in this study were purchased from Invitrogen (China). Stock solutions of all the derivatives (10 mM) were made using DMSO (10%) or double-distilled deionized water. Further dilutions to working concentrations were made with double-distilled deionized water.

### 5.3. FRET-melting assay

FRET assay was carried out following previously reported procedures [44]. The fluorescently labeled oligomer FPu18T (5′-FAM-AGGGTGGGGA-GGGTGGGG-TAMRA-3′) and F10T [5′-FAM-dTATAGCTATA-HEG-TATA-GCTATA-TAMRA-3′] (HEG linker: [(−$CH_2$−$CH_2$−O−)$_6$]) were used as G-quadruplex and duplex DNA model respectively. The experiments were performed in real-time PCR apparatus (Roche LigntCycler), allowing the simultaneous recording of 32 samples. The melting of each sample, which containing 0.2 μM oligonucleotide FPu18T or F10T in 10 mM Tris–HCl buffer, pH 7.2, 0.2 mM KCl, was monitored in the presence or absence of 2 μM compounds by measuring their fluorescence intensity with excitation at 470 nm and detection at 530 nm, and the experiment was repeated for three times. Fluorescence readings were taken at an interval of 1 °C over the range 37–99 °C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. Final analysis of the data was carried out using Origin 8.0 (OriginLab Corp.).

To test the binding selectivity of the compounds for their interactions with the G-quadruplex DNA over duplex DNA, we added various concentrations of double-stranded DNA (self-complementary ds26 DNA: 5′-GTTAGCCTAGCTTAAGCTAGGCTAAC-3′) as competitor. The $T_{1/2}$ was defined as the temperature value when the normalized fluorescence emission is 0.5.

### 5.4. Surface plasmon resonance

SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a Neutravidin-coated GLH sensor chip [46,47]. In a typical experiment, biotinylated Pu27 was dissolved in filtered and degassed running buffer (50 mM Tris–HCl, pH 7.2, 100 mM KCl). The DNA samples were then captured ($\sim$1000 RU) in flow cells, leaving the last flow cell as a blank. Ligand solutions (at 10, 5, 2.5, 1.25, 0.625, 0.3125 μM) were prepared with running buffer through serial dilutions from stock solution. Six concentrations were injected simultaneously at a flow rate of 100 μL min$^{-1}$ for 400 s of association phase, followed with 400 s of dissociation phase at 25 °C. The GLH sensor chip was regenerated with short injection of 50 mM NaOH between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams from G-quadruplex sensorgrams. Data were analyzed with ProteOn manager software, using the Equilibrium method for fitting kinetic data.

### 5.5. CD measurements

CD experiments were performed on a chirascan circular dichroism spectrophotometer (Applied Photophysics) [21]. A quartz cuvette with 1 cm path length was used for the spectra recorded over a wavelength range of 230–430 at 1 nm bandwidth, 1 nm step size, and 0.5 s per point. 1 μM of the oligomer Pu27 (5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′) in 10 mM Tris–HCl buffer, pH 7.2, 100 mM KCl was annealed by heating at 95 °C for 5 min, and then gradually cooled to room temperature and incubated at 4 °C overnight. The CD titration experiment was performed at a fixed Pu27 concentration (1 μM) by adding various concentrations (0–10 μmol) of the ligands in Tris–HCl buffer, 100 mM KCl at 25 °C. After each addition of ligand, the reaction was stirred and allowed to equilibrate for at least 10 min (until no elliptic changes were observed), and then a CD spectrum was collected by averaging two scans. A buffer baseline was collected in the same cuvette, and subtracted from the sample spectra. Final analysis of the data was carried out using Origin 8.0 (OriginLab Corp.).

The CD experiment of oligomer Pu27 DNA was also carried out at a final concentration of 1 μM in 10 mM Tris–HCl buffer, pH 7.2 at 25 °C containing the tested ligands (0, 3, 5, and 10 μM) in the absence of KCl. The samples containing Pu27 and various concentrations of compounds in 10 mM Tris–HCl buffer, pH 7.2, were annealed by heating at 95 °C for 5 min, and then gradually cooled to room temperature and incubated at 4 °C overnight. The recording of CD spectra was carried out following the titration experiment.

### 5.6. PCR-stop assay

The PCR-stop assay was performed with a modified protocol of a previously reported procedure [24]. The oligomer of Pu27 (5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′) and its corresponding complementary sequence (5′-ATCGATCGCTTCTCGTCCTTCCCCA-3′) were used in the experiment. The reactions were performed in 1 × PCR-stop buffer, containing 10 μM of each pair of oligomers, 0.16 μM of dNTP, 2.5 U of *Taq* polymerase, and the indicated amount of the compounds. Reaction mixtures were incubated in a thermocycler, with the following cycling conditions: 94 °C for 2 min, followed with 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Amplified products were loaded on 16% non-denaturing polyacrylamide gel in 1 × TBE and GelRed stained.

### 5.7. Molecular modeling

Pu-18B G-quadruplex DNA structure constructed as reported previously [45] was used as an initial model to study the interaction between 5,6-dihydrobenzo[*c*]acridine derivatives and human *c-myc* G-quadruplex DNA. The ligands were firstly constructed and minimized in SYBYL 7.3.5 (Tripos Inc., St. Louis, MO, USA). Docking studies were carried out using the AUTODOCK 4.2 program. The G-quadruplex structure was used as an input for the AUTOGRID program. The grid box was placed at the center of the G-quadruplex. The dimensions of the active site box were set at 60 × 60 × 60 Å. Docking calculations were carried out using the Lamarckian genetic algorithm (LGA). Other parameters were used according to the default. Hundred of independent docking runs were carried out. The ligand conformation was chosen based on both the docked energy and the RMSD (root-mean-square-deviation).

### 5.8. Proliferation assay

The Ramos cell line and CA46 cell line were used in the short-term proliferation experiments. Cells were seeded in 24-well plates at $1.0 \times 10^5$ per well and exposed to the indicated concentrations of the ligand or an equivalent volume of 0.1% DMSO. The cells in control and drug-exposed wells were counted every day and the experiment was carried out for 4 days.

### 5.9. RNA extraction

Cell pellets harvested from each well of the culture plates were lysed in TRIpure solution. RNA was extracted with M-MLV according to manufacturer's protocol and eluted in distilled, deionized water with 0.1% diethyl pyrocarbonate (DEPC) to a final volume of 10–50 mL. RNA was quantitated spectra-photometrically and stored at −80 °C.

### 5.10. RT-PCR

Total RNA was used as a template for reverse transcription using the following protocol: each 20 μL reaction contained 5 × M-MLV buffer, 2.5 mM dNTP, 100 pM oligo $dT_{18}$ primer, 1 μL M-MLV reverse transcriptase, DEPC in water (DEPC $H_2O$), and 2 μg of total RNA. Briefly, RNA and oligo $dT_{18}$ primer were incubated at 70 °C for 10 min, and then immediately placed on ice. Next, the other components were added, and incubated at 42 °C for 1 h, and then at 70 °C for 15 min. Finally, the reacted solution was stored at −20 °C. Both *c-myc* and β-actin were amplified by using a real-time PCR apparatus (Roche LightCycler 2), and the PCR products were analyzed with electrophoresis on 1% agarose gel at 120 V for 20 min.

## Conflict of interest

We declare that we have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.03.034.

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