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Bisacridines with aromatic linking chains. Synthesis, DNA interaction, and antitumor activity

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Abstract—Synthesis of a series of bisacridine derivatives containing rigid aromatic linking chains is described. Their DNA interaction and in vitro cytotoxicity against HT-29 human carcinoma cells are reported.

1. Introduction

Flat aromatic or heteroaromatic molecules bind to DNA by inserting, intercalating, and stacking between the base pairs of the double helix. The principal driving forces for intercalation are stacking and charge–transfer interactions, although hydrogen bonding and electrostatic forces also play an important role in stabilization.[1,2] In general, intercalation does not disrupt the Watson–Crick hydrogen bonding. It does, however, destroy the regular helical structure, unwinding the DNA at the site of binding and, as a result of this, it interferes with the action of DNA-binding enzymes such as DNA topoisomerases and DNA polymerases.

Simple acridine intercalators, including proflavine (3,6-diaminoacridine) bind to DNA with their entire structure inserted between and parallel with the base pairs, with no binding in the grooves.[3,4] Footprinting studies have shown that proflavine binds best to mixed sequences of alternating pyrimidines and purines and is excluded from runs of A and T. These cationic intercalators interact with DNA through nonspecific electrostatic binding with the phosphate backbone and diffuse along the surface until they encounter gaps between base pairs sufficient for intercalation.[5]

In the last years the synthesis of bisintercalators has aroused considerable attention because, compared with monointercalators, higher DNA-binding constants, slower dissociation rates, and substantial sequence selectivity can be expected by incorporating two or more intercalating units in a polyfunctional ligand. In addition, groove or phosphate interactions of the linker joining the two intercalating chromophores may provide enhancement for binding affinity or selectivity. Thus, several bisintercalators have been synthesized employing the chromophores of acridine connected with flexible and rigid linkers of various lengths.[6,7] Compounds containing rigid linkers are often preferred over those containing more flexible linking chains. The latter exhibit lower affinities toward DNA since entropic effects and self-stacking interactions compete with the binding process.[8,9] On the other hand, flexible bisintercalators show reduced lifetimes at any given site, which have been correlated with lower in vivo antitumor activities.[10,11]

In preliminary communications[12,13] we described the synthesis and antitumor activity of several bisacridines with rigid chains as linkers (Scheme 1). The cytotoxicity of these compounds was evaluated by measuring the growth inhibition of P-388, A-549, HT-29, and MEL-28 cells. Deprotection of the amino group could increase the solubility of these compounds in an aqueous medium and facilitate the interaction with nucleic acids by establishing hydrogen bonds with the phosphate groups. However, usual methods for cleavage of the methoxycarbonyl group were unsuccessful.

We report now the synthesis of five new bisacridine derivatives tethered to rigid aromatic linking chains of different length (from 7.0 to 9.6 Å). These lengths allow, in all compounds, except for compound **4c**, for a

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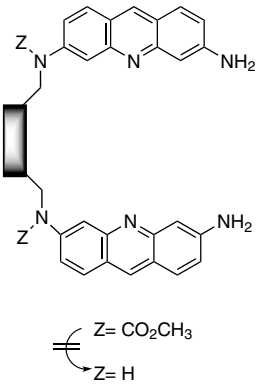


Scheme 1.

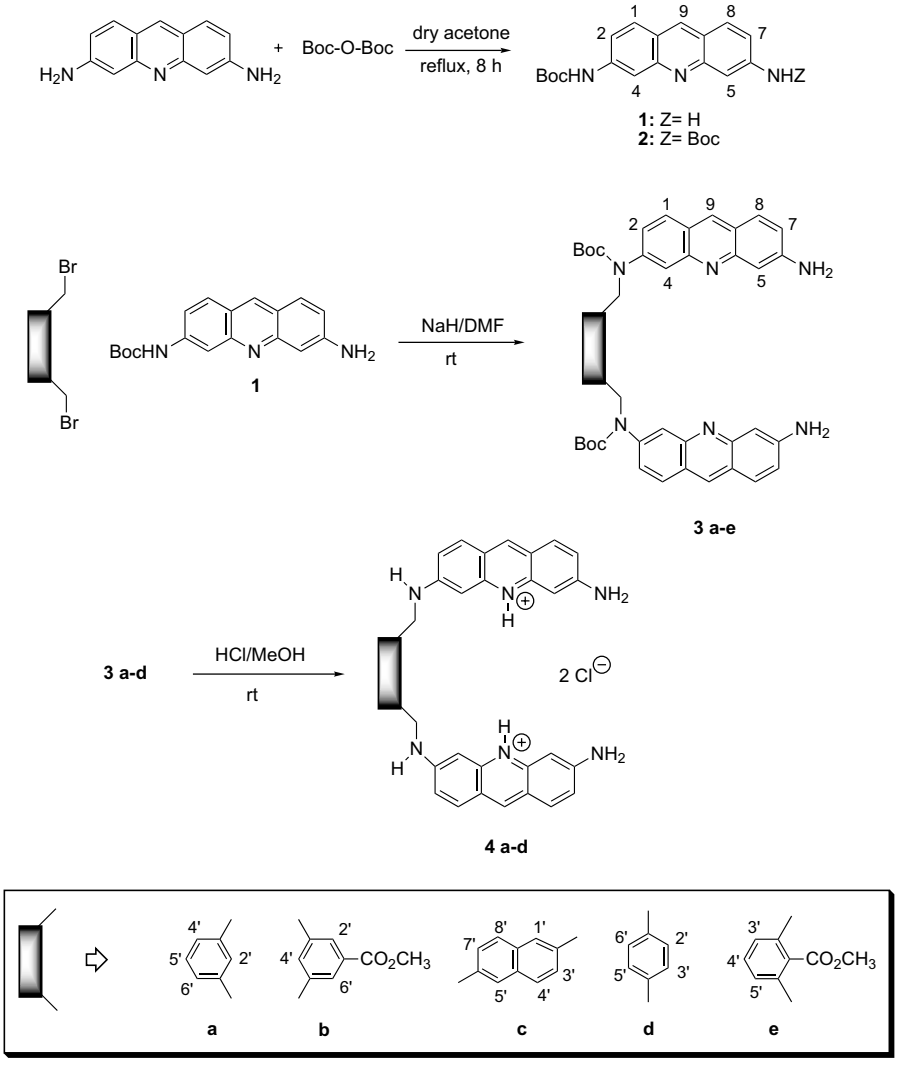
monointercalation or bisintercalation process, with violation of the neighbor exclusion principle. For compound **4c**, in which the chromophores are separated at the highest distance, a bisintercalation taking place in accordance with the neighbor exclusion principle is also feasible. Moreover, some of the aromatic chains possess groups that might facilitate DNA interaction acting as hydrogen bond acceptors.

This time, deprotection of the amino group is easily accomplished by choosing the *tert*-butoxycarbonyl moiety as the protecting group.

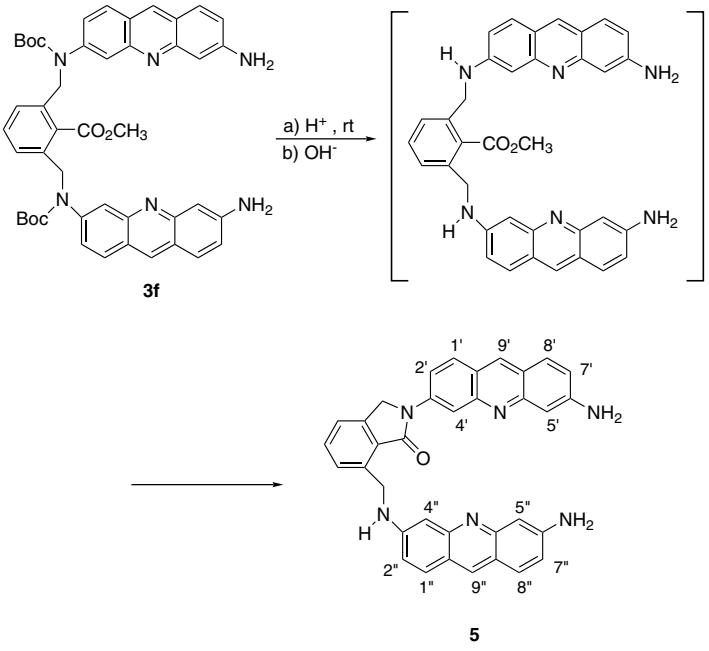
2. Synthesis

As it is shown in Scheme 2, the first step of the synthetic route consisted of the reaction between 3,6-diaminoacridine (proflavine) and di-*tert*-butyl dicarbonate to give (6-amino-3-acridinyl)carbamic acid *tert*-butyl ester (**1**). The dicarbamate **2** was also obtained as a by-product.

Subsequent reaction of the monocarbamate **1**, as sodium salt, with one-half equivalent of a benzylic bromide in DMF at room temperature afforded the bisacridines **3 a–e** (Scheme 2). The *tert*-butoxycarbonyl protecting groups were easily removed by treating **3** with hydrogen chloride in methanol to provide the compounds **4** as dihydrochlorides.



Scheme 2.



Scheme 3.

The structure of the synthesized bisacridines **3** and **4** was established from ^{1}H NMR, mass spectra, and microanalytical data (see Section 4). The hydrogens 2 and 4 of bisacridines **3** were shifted to higher field compared with the same hydrogens of monocarbamate **1**, which can be attributed to the anisotropic shielding effect of the aromatic ring in the spacer.

For bisintercalant **3e**, deprotection in acidic medium unexpectedly afforded 2-(6-amino-3-acridinyl)-7-[(6-amino-acridin-3-ylamino)methyl]-2,3-dihydroisoindol-1-one (**5**) resulting from cyclization of the amino with the ester group (Scheme 3).

The ^{1}H NMR (DMSO-d_6) spectrum of **5** showed the signals of hydrogens 2 and 7 of the acridine ring as a doublet of doublets (J = 9.2 and 1.8 Hz) by coupling with the hydrogens 1,4 and 5,8, respectively. Correspondingly, the signals of hydrogens 1 and 8 appeared as doublets and those of hydrogens 4 and 5 as doublets or broad singlets. One of the methylene groups was observed as a singlet and the other one as a doublet by coupling with the N–H. This coupling disappeared by addition of D_2O. The unusually high chemical shift of the hydrogen 4′ can be explained by the anisotropic effect of the carbonyl group of the isoindolone ring.

3. DNA-binding studies and biological activity

Addition of compounds **4a**–**d** to a solution of calf thymus DNA in 10 mM sodium phosphate buffer ([NaCl] = 15 mM, pH = 5.6) gave rise to hypochromic and bathocromic shifts on complex formation. Relative to the free-compound absorption spectra, the major ultraviolet bands in **4a**–**d** bound to calf thymus DNA were red-shifted and displayed hypochromic effects for the absorptions at 437 and 445 nm (Table 1). Unfortunately, the hypochromicities in the absorption maxima for compounds **4a**–**d** were low and that prevented accurate determination of their DNA-binding constants.

In vitro cytotoxicity was determined against exponentially growing HT-29 human colon carcinoma cells in culture. The cells were exposed to drug for 72 h at 37 °C. Table 1 shows the IC_{50} (μM) values calculated as the concentration of drug needed to inhibit cell growth in 50% of controls.

It is apparent from Table 1 that the interaction between calf thymus DNA and the synthesized bisacridines seems to correlate well with biological activities in HT-29 cell line. Thus, compounds that showed a higher interaction (expressed as %H) gave rise to lower IC_{50} values.

4. Experimental

All melting points were determined in open capillary on a Electrothermal IA 9100 and are uncorrected. UV–vis measurements were performed on a Lambda 18 Perkin–Elmer spectrometer. NMR spectra were recorded on a Varian Unity at 300 or 500 MHz. Mass spectra by electronic impact were registered on a Hewlett–Packard HP-5988 at 70 eV and FAB mass spectra on a V.G. Autoexpec in a 3-nitrobenzyl alcohol matrix. Microanalyses were performed in a Heraeus CHN. Flash column chromatographies were carried out on silica gel SDS 230–400 mesh. Calf thymus DNA for spectrophotometric binding analyses was purchased

Table 1. DNA interaction and cytostatic activity of bisacridines **4a–d**

Compounds	λ_{free}	λ_{bound}	ε_{free}	ε_{bound}	%H[a]	Cytotoxicity IC_{50} (μM)
4a	439	466	37,820	29,455	22	8.9
4b	437	465	34,835	29,500	15	9.7
4c	445	465	21,085	18,550	12	17
4d	446	466	15,365	15,116	2	26

[a] %H = percent hypochromicity [$\%H = (1-\varepsilon_{bound}/\varepsilon_{free})\times 100$].

from the Sigma–Aldrich Co., 1,2- and 1,3-bis(bromomethyl)benzene were purchased from Aldrich and used without further purification. Preparation of methyl 3,5-bis(bromomethyl)benzoic acid methyl ester,[14] methyl 2,6-bis(bromomethyl)benzoic acid methyl ester,[15] and 2,6-bis(bromomethyl)naphthalene[16] was performed according to reported procedures.

4.1. Reaction of 3,6-diaminoacridine with di-*tert*-butyldicarbonate: synthesis of carbamates 1 and 2

To a solution of di-*tert*-butyldicarbonate (4.4 mL, 19.12 mmol) in dry acetone (150 mL), 3,6-diaminoacridine (2 g, 9.56 mmol) was added. The reaction mixture was heated at reflux for 8 h and then concentrated up to dryness. The crude product thus obtained was purified by flash column chromatography using silica gel as adsorbent. With hexane–ethyl acetate (1:1) as eluent 3,6-acridinediylbiscarbamic acid di-*tert*-butyl ester (**2**) (746 mg, 25%) was obtained; mp 163–165 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.88 (s, 18H, CO_2-*t*-Bu), 7.56 (dd, 2H, J = 9.1, 2.2 Hz, H-2,7), 7.94 (d, 2H, J = 9.1 Hz, H-1,8), 8.19 (d, 2H, J = 2.2 Hz, H-4,5), 8.74 (s, 1H, H-9), and 9.8 (s, 2H, N–H). EI-MS *m*/*z* : 409 (M^+, 17%), 353 (8), 309 (18), 297 (93), 254 (13), 253 (83), 235 (3), 210 (11), 209 (72), 208 (25), 182 (31), 181 (37), 179 (11), 154 (13), 153 (11), 127 (9). Anal. Calcd for $C_{23}H_{27}N_3O_4$: C, 67.46; H, 6.65; N, 10.26. Found: C, 67.59; H, 6.53; N, 10.02.

Continued elution with ethyl acetate and then ethyl acetate–acetone–triethylamine (5:5:1) afforded (2.3 g, 59%) of the (6-amino-3-acridinyl)carbamic acid *tert*-butyl ester (**1**); mp > 280 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.75 (s, 9H, CO_2-*t*-Bu), 5.77 (br s, 2H, NH_2), 6.83 (d, 1H, J = 2.0 Hz, H-5), 6.97 (dd, 1H, J = 8.9, 2.2 Hz, H-7), 7.42 (dd, 1H, J = 8.9, 2.2 Hz, H-2), 7.72 (d, 1H, J = 8.9 Hz, H-8), 7.8 (d, 1H, J = 8.9 Hz, H-1), 8.03 (d, 1H, J = 2.2 Hz, H-4), 8.5 (s, 1H, H-9), 9.67 (s, 1H, N–H). EI-MS *m*/*z*: 309 (M^+, 8%), 253 (33), 235 (23), 209 (100), 182 (31), 181 (25), 127 (5), 104 (7), 57 (10). Anal. Calcd for $C_{18}H_{19}N_3O_2$: C, 69.88; H, 6.19; N, 13.58. Found: C, 69.61; H, 6.13; N, 13.84.

4.2. Synthesis of bisacridines 3. General procedure

To a suspension of 80% sodium hydride (26 mg, 0.86 mmol) in dry DMF (20 mL) under argon, (6-amino-3-acridinyl)carbamic acid *tert*-butyl ester (240 mg, 0.77 mmol) was added. The reaction mixture was stirred at room temperature for 20 min and then the corresponding benzylic bromide (0.39 mmol) was added. The mixture was maintained with stirring at room temperature and worked-up according the procedure of each particular case.

4.3. [1,3-Phenylenebis(methylene)]bis[(6-amino-3-acridinyl)]carbamic acid di-*tert*-butyl ester (3a)

According to the general procedure and after 30 min with stirring at room temperature, the solvent was evaporated in vacuo and the resulting residue was dissolved in dichloromethane and washed with water. The organic phase was dried ($MgSO_4$) and concentrated up to dryness. The crude product thus obtained was purified by flash column (diameter: 3 cm) chromatography using silica gel as adsorbent and ethyl acetate–acetone–triethylamine (5:2:1) as eluent to afford **3a** (208 mg, 75%); mp 142 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.24 (s, 18H, CO_2-*t*-Bu), 4.95 (s, 4H, CH_2), 6.09 (br s, 4H, NH_2), 6.85 (d, 2H, J = 1.8 Hz, H-5 acridine), 7.05 (dd, 2H, J = 8.8, 1.83 Hz, H-7 acridine), 7.01–7.11 (m, 2H, H-4′,6′), 7.14–7.24 (m, 2H, H-2′,5′), 7.16 (dd, 2H, J = 8.8, 1.8 Hz, H-2 acridine), 7.61 (d, J = 1.8 Hz, 2H, H-4 acridine), 7.69 (d, 2H, J = 8.8 Hz, H-8 acridine), 7.75 (d, 2H, J = 8.8 Hz, H-1 acridine), 8.49 (s, 2H, H-9 acridine). FAB-MS *m*/*z*: 721 (M^++1, 71%). Anal. Calcd for $C_{44}H_{44}N_6O_4$: C, 73.31; H, 6.15; N, 11.66. Found: C, 73.61; H, 6.02; N, 11.49.

4.4. *N*,*N*′-Bis(6-amino-3-acridinyl)-*N*,*N*′-bis(*tert*-butoxycarbonyl)-3,5-bis(aminomethyl)benzoic acid methyl ester (3b)

Following the general procedure and after 4 h at room temperature, the reaction mixture was concentrated in vacuo. The solid residue was purified by flash column (diameter: 3 cm) chromatography using silica gel as adsorbent and ethyl acetate–acetone–triethylamine (5:5:1) as eluent to afford **3c** (51 mg, 17%); mp 180 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.31 (s, 18H, CO_2-*t*-Bu), 3.76 (s, 3H, CO_2CH_3), 5.04 (s, 4H, CH_2), 6.1 (br s, 4H, NH_2), 6.85 (d, 2H, J = 2.2 Hz, H-5 acridine), 7.05 (dd, 2H, J = 9.2, 2.2 Hz, H-7 acridine), 7.16 (dd, 2H, J = 9.2, 2.2 Hz, H-2 acridine), 7.47 (br s, 1H, H-4′), 7.61 (d, 2H, J = 2.2 Hz, H-4 acridine), 7.70 (d, 2H, J = 9.2 Hz, H-8 acridine), 7.75 (d, 2H, J = 1.5 Hz, H-2′,6′), 7.76 (d, 2H, J = 9.2 Hz, H-1 acridine), 8.49 (s, 2H, H-9 acridine). FAB-MS: *m*/*z*: 779 (M^++1, 69%). Anal. Calcd for $C_{46}H_{46}N_6O_6$: C, 70.93; H, 5.95; N, 10.79. Found: C, 70.65; H, 6.13; N, 11.12.

4.5. [2,6-Naphthalenediylbis(methylene)]bis[(6-amino-3-acridinyl)]carbamic acid di-*tert*-butyl ester (3c)

According to the general procedure and after 10 h with stirring at room temperature, the solvent was evaporated in vacuo and the resulting residue was purified by column (diameter: 2 cm) chromatography using silica gel as adsorbent and ethyl acetate–triethylamine (40:1) as eluent to afford **3c** (141 mg, 48%); mp 142 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.38 (s, 18H, CO_2-*t*-Bu), 5.12 (s, 4H, CH_2), 6.09 (br s, 4H, NH_2), 6.8 (d, 2H, J = 1.8 Hz, H-5 acridine), 7.01 (dd, 2H, J = 8.9, 1.8 Hz, H-7 acridine), 7.28 (dd, 2H, J = 8.6, 1.8 Hz, H-2 acridine), 7.39 (d, 2H, J = 7.7 Hz, H-3′,7′), 7.65–7.67 (m, 4H, H-4 acridine and H-1′,5′), 7.74 (d, 2H, J = 8.9 Hz, H-8 acridine), 7.78–7.8 (m, 4H, H-1 acridine and H-4′,8′), 8.55 (s, 2H, H-9 acridine). FAB-MS *m*/*z*: 771 (M^++1, 72%). Anal. Calcd for $C_{48}H_{46}N_6O_4$: C, 74.78; H, 6.01; N, 10.9. Found: C, 74.61; H, 6.15; N, 10.72.

4.6. [1,4-Phenylenebis(methylene)]bis[(6-amino-3-acridinyl)]carbamic acid di-*tert*-butyl ester (3d)

After 2 h with stirring at room temperature, the reaction mixture was concentrated at reduced pressure and the residue thus obtained was dissolved in dichloromethane and washed with water. The organic phase was dried ($MgSO_4$) and concentrated up to dryness. The resulting solid was purified by flash column (diameter: 3 cm) chromatography using silica gel as adsorbent and ethyl acetate–acetone–triethylamine (5:2:1) as eluent to afford **3d** (161 mg, 58%); mp 178 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.27 (s, 18H, CO_2-*t*-Bu), 4.93 (s, 4H, CH_2), 6.09 (br s, 4H, NH_2), 6.83 (d, 2H, J = 1.8 Hz, H-5 acridine), 7.04 (dd, 2H, J = 9.2, 1.8 Hz, H-7 acridine), 7.18 (s, 4H, H-2′,3′,5′,6′), 7.2 (dd, 2H, J = 9.2, 2.2 Hz, H-2 acridine), 7.59 (d, 2H, J = 2.2 Hz, H-4 acridine), 7.76 (d, 4H, J = 9.2 Hz, H-1,8 acridine), 8.53 (s, 2H, H-9 acridine). FAB-MS *m*/*z*: 721 (M^++1, 58%). Anal. Calcd for $C_{44}H_{44}N_6O_4$: C, 73.31; H, 6.15; N, 11.66. Found: C, 73.5; H, 6.21; N, 11.29.

4.7. *N*,*N*′-Bis(6-amino-3-acridinyl)-*N*,*N*′-bis(*tert*-butoxycarbonyl)-2,6-bis(aminomethyl)benzoic acid methyl ester (3e)

According to the general procedure and after 3 h with stirring at room temperature, the solvent was evaporated at reduced pressure. The crude product was purified by flash column (diameter: 3 cm) chromatography using silica gel as adsorbent and ethyl acetate–acetone–triethylamine (4:3:1) as eluent to afford **3e** (225 mg, 75%); mp 190–195 °C (hexane–dichloromethane). ^{1}H NMR (DMSO-d_6) δ 1.31 (s, 18H, CO_2-*t*-Bu), 3.73 (s, 3H, CO_2CH_3), 4.97 (s, 4H, CH_2), 6.1 (br s, 4H, NH_2), 6.83 (s, 2H, H-5 acridine), 7.03 (d, 2H, J = 9.2, H-7 acridine), 7.19 (d, 2H, J = 9.2, H-2 acridine), 7.21 (d, 2H, J = 7.69 Hz, H-3′,5′), 7.3 (t, 1H, J = 7.7 Hz, H-4′), 7.57 (br s, 2H, H-4 acridine), 7.74 (d, 4H, J = 9.2 Hz, H-1,8 acridine), 8.51 (s, 2H, H-9 acridine). FAB-MS *m*/*z*: 779 (M^++1, 100%). Anal. Calcd for $C_{46}H_{46}N_6O_6$: C, 70.93; H, 5.95; N, 10.79. Found: C, 71.2; H, 5.81; N, 10.52.

4.8. Deprotection of bisacridines 3. General procedure for preparation of bisacridines 4

A solution of the corresponding bisacridine **3** (0.6 mmol) in methanol (20 mL) saturated with hydrogen chloride, was stirred at room temperature for 15 min. Then, the reaction mixture was concentrated up to dryness and the resulting residue tritured with dichloromethane and filtered.

4.9. *N*,*N*′-Bis(6-amino-3-acridinyl)-1,3-benzenedimethanamine dihydrochloride (4a)

Yield: 92%; mp >300 °C. ^{1}H NMR (DMSO-d_6) δ 4.47 (s, 4H, CH_2), 6.7 (br s, 2H, H-5 or H-4 acridine), 6.78 (d, 2H, J = 1.8 Hz, H-4 or H-5 acridine), 6.95 (dd, 2H, J = 9.1, 1.8 Hz, H-7 or H-2 acridine), 7.06 (dd, 2H, J = 9.1, 1.8 Hz, H-2 or H-7 acridine), 7.24–7.41 (m, 3H, H-4′,5′,6′), 7.5 (br s, 1H, H-2′), 7.68 (d, 2H, J = 9.1 Hz, H-8 or H-1 acridine), 7.74 (d, 2H, J = 9.1 Hz, H-1 or H-8 acridine), 8.47 (br s, 2H, N–H), 8.6 (s, 2H, H-9 acridine), 14.06 (s, 2H, N^+–H). Anal. Calcd for $C_{34}H_{30}Cl_2N_6$: C, 68.8; H, 5.09; N, 14.16. Found: C, 68.61; H, 5.12; N, 14.4.

4.10. *N*,*N*′-Bis(6-amino-3-acridinyl)-3,5-bis(aminomethyl)benzoic acid methyl ester dihydrochloride (4b)

Yield: 68%; mp >300 °C. ^{1}H NMR (DMSO-d_6) δ 3.81 (s, 3H, CO_2CH_3), 4.56 (d, 4H, J = 5.5 Hz, CH_2), 6.69 (br s, 2H, H-5 or H-4 acridine), 6.78 (br s, 2H, H-4 or H-5 acridine), 6.96 (d, 2H, J = 9.2 Hz, H-7 or H-2 acridine), 7.09 (d, 2H, J = 9.2, H-2 or H-7 acridine), 7.69 (d, 2H, J = 9.2 Hz, H-8 or H-1 acridine), 7.74 (d, 2H, J = 9.2 Hz, H-1 or H-8 acridine), 7.81 (s, 1H, H-4′), 7.89 (s, 2H, H-2′,6′), 8.53 (t, 2H, J = 5.5 Hz, N–H), 8.6 (s, 2H, H-9 acridine), 14.07 (s, 2H, N^+–H). Anal. Calcd for $C_{36}H_{32}Cl_2N_6O_2$: C, 66.36; H, 4.95; N, 12.9. Found: C, 66.61; H, 5.13; N, 12.58.

4.11. *N*,*N*′-Bis(6-amino-3-acridinyl)-2,6-naphthalenedimethanamine dihydrochloride (4c)

Yield: 95%; mp >300 °C. ^{1}H NMR (DMSO-d_6) δ 4.63 (s, 4H, CH_2), 6.7–6.76 (m, 4H, H-4,5 acridine), 6.95 (dd, 2H, J = 9.0, 2.2 Hz, H-7 or H-2 acridine), 7.12 (dd, 2H, J = 9.2, 2.2 Hz, H-2 or H-7 acridine), 7.54 (d, 2H, J = 9.9 Hz, H-3′,7′), 7.77–7.82 (m, 4H, H-8 or H-1 acridine and H-1′,5′), 7.91–7.93 (m, 4H, H-1 or H-8 acridine and H-4′,8′), 8.49 (br s, 2H, N–H), 8.72 (s, 2H, H-9 acridine), 13.98 (s, 2H, N^+–H). Anal. Calcd for $C_{38}H_{32}Cl_2N_6$: C, 70.91; H, 5.01; N, 13.06. Found: C, 71.13; H, 4.96; N, 12.83.

4.12. *N*,*N*′-Bis(6-amino-3-acridinyl)-1,4-benzenedimethanamine dihydrochloride (4d)

Yield: 91%; mp >300 °C. ^{1}H NMR (DMSO-d_6) δ 4.47 (s, 4H, CH_2), 6.68 (d, 2H, J = 2.7 Hz, H-5 or H-4 acridine), 6.74 (d, 2H, J = 2.7 Hz, H-4 or H-5 acridine), 6.95 (dd,

2H, $J=9.1$, 2.7 Hz, H-7 or H-2 acridine), 7.08 (dd, 2H, $J=9.1$, 2.7 Hz, H-7 or H-2 acridine), 7.41 (s, 4H, benzenic), 7.79 (d, 2H, $J=9.1$ Hz, H-8 or H-1 acridine), 7.8 (d, 2H, $J=9.1$ Hz, H-1 or H-8 acridine), 8.38 (br s, 2H, N–H), 8.73 (s, 2H, H-9 acridine), 13.91 (s, 2H, N^+-H). Anal. Calcd for $C_{34}H_{30}Cl_2N_6$: C, 68.8; H, 5.09; N, 14.16. Found: C, 69.12; H, 4.91; N, 15.42.

4.13. Deprotection of 3e. Synthesis of 2-(6-amino-3-acridinyl)-7-[(6-amino-acridin-3-ylamino)-methyl]-2,3-dihydroisoindol-1-one (5)

A solution of **3e** (100 mg, 0.128 mmol) in trifluoroacetic acid (10 mL) was stirred at room temperature for 10 min. The reaction mixture was basified with 20% sodium hydroxide and the precipitate thus obtained was filtered and washed with cold water. The crude product was purified by flash column (diameter: 2 cm) chromatography using silica gel as adsorbent and ethyl acetate–methanol–triethylamine (5:1:1) as eluent affording pure **5** (42 mg, 61%). mp >300 °C. ^{1}H NMR (DMSO-d_6) δ 5.1 (d, 2H, $J=5.86$ Hz, NH–CH_2), 5.26 (s, 2H, N–CH_2), 6.57 (br s, 1H, H-5′ or 5″), 6.63 (d, 1H, $J=1.8$ Hz, H-5″ or 5′), 6.83 (d, 1H, $J=1.8$ Hz, H-4″), 6.96 (dd, 1H, $J=9.1$, 1.8 Hz, H-7′ or 7″), 7.16 (dd, 1H, $J=9.2$, 1.8 Hz, H-7″ or 7′), 7.2 (dd, 1H, $J=9.2$, 1.8 Hz, H-2′ or 2″), 7.31 (br s, 2H, NH_2), 7.48 (d, 1H, $J=6.6$ Hz, benzenic), 7.65–7.73 (m, 2H, benzenic), 7.82 (d, 1H, $J=9.2$ Hz, H-8′ or 8″), 7.86 (d, 1H, $J=9.16$ Hz, H-8″ or 8′), 7.92 (br s, 2H, NH_2), 8.03 (d, 1H, $J=9.2$ Hz, H-1″ or 1′), 8.09 (dd, 1H, $J=9.2$, 1.8, H-2′ or 2″), 8.30 (d, 1H, $J=9.2$ Hz, H-1′ or 1″), 8.38 (t, 1H, $J=5.9$ Hz, N–H), 8.79 (s, 1H, H-9′ or 9″), 8.84 (br s, 1H, H-4′), 9.16 (s, 1H, H-9″ or 9′). FAB-MS *m*/*z*: 547 (M^++1, 44%). Anal. Calcd for $C_{35}H_{26}N_6O$: C, 76.9; H, 4.79; N, 15.37. Found: C, 76.73; H, 4.62; N, 15.65.

4.14. In vitro cytotoxicity evaluation

The cytotoxicity was measured using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human colon carcinoma cell line HT-29 was obtained from American Type Culture Collection and cultured in the recommended media. Exponentially growing cells were plated at 3000/well into 96-well plates in 150 μL complete DMEM media containing 10% FBS. Cells were allowed to attach for 24 h before the addition of a serial (1:4) dilution of drug in 50 μL fresh media. After 72 h of incubation at 37 °C, 5% CO_2, MTT (50 μL: 3 mg/mL in PBS) was added to each well and the plates incubated for 4 h. Formazan crystals formed by MTT metabolism were solubilized by the addition of 50 μL of 25% SDS pH 2 to each well and incubating overnight. The cellular metabolism of MTT was then quantified by reading the absorbance of the solubilized product at 550 nm with a 96-well plate reader.

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