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Potent antitumor 9-anilinoacridines bearing an alkylating N-mustard residue on the anilino ring: synthesis and biological activity

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Abstract—A series of N-mustard derivatives of 9-anilinoacridine was synthesized for antitumor and structure–activity relationship studies. The alkylating N-mustard residue was linked to the C-3′ or C-4′ position of the anilino ring with an *O*-ethylene (O–C_2), *O*-butylene (O–C_4), and methylene (C_1) spacer. All of the new N-mustard derivatives exhibited significant cytotoxicity in inhibiting human lymphoblastic leukemic cells (CCRF–CEM) in culture. Of these agents, (3-(acridin-9-ylamino)-5-{2-[bis(2-chloroethyl)amino]ethoxy}phenyl)methanol (**10**) was subjected to antitumor studies, resulting in an approximately 100-fold more potent effect than its parent analogue 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA) in inhibiting the growth of human lymphoblastic leukemic cells (CCRF–CEM) in vitro. This agent did not exhibit cross-resistance against vinblastine-resistant (CCRF–CEM/VBL) or Taxol-resistant (CCRF–CEM/Taxol) cells. Remarkably, the therapeutic effect of **10** at a dose as low as one tenth of the Taxol therapeutic dose [i.e., 1–2 mg/kg (Q3D × 7) or 3 mg/kg (Q4D × 5); intravenous injection] on nude mice bearing human breast carcinoma MX-1 xenografts resulted in complete tumor remission in two out of three mice. Furthermore, **10** yielded xenograft tumor suppression of 81–96% using human T-cell acute lymphoblastic leukemia CCRF–CEM, colon carcinoma HCT-116, and ovarian adenocarcinoma SK-OV-3 tumor models.

1. Introduction

The alkylating nitrogen mustard derivatives exert their cytotoxic effects by interstrand cross-linking of DNA.[1] Sequences with contiguous guanines have the lowest molecular electrostatic potentials and therefore are more vulnerable to nucleophilic attacks.[2,3] Earlier works on the isolation of cross-linked fragments from digested alkylator-treated DNA showed that these cross-links occurred predominantly on the guanine N7 position.[4] However due to the formation of mono-alkylation or interaction with other cellular components via partial hydrolysis, the high reactivity of these agents lack the affinity to bind to DNA, resulting in the loss of their antitumor activity. The prevalence of forming the mono-alkylation has proven to produce genotoxicity and resulted in less cytotoxicity.[5]

Simple alkylators have little ability to recognize sequences larger than a single nucleotide.[6] Additionally, resistance to such reactive electrophiles is easily developed by an increase in the cellular levels of low-molecular-weight thiol (particularly glutathione).[7] In principle, many of these drawbacks could be ameliorated by linking the N-mustard to a carrier molecule having an affinity for DNA, which by reacting with other cell components should result in less diversion of the active drug. The use of sequence-specific carriers should direct the pattern of alkylation sites on the DNA and ultimately provide specific targets to certain genes. An

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improvement in the proportion of lethal cross-linking with respect to more genotoxic monoalkylation events may limit the known carcinogenicity of alkylators.[8] Finally, the development of resistance to such compounds by elevating cellular thiol levels may be of less concern.

Thus, designing and synthesizing DNA-directed alkylators have been one of the attractive approaches in the development of potent anticancer drugs. Earlier works on the development of DNA-directed alkylating agents have employed DNA-intercalating chromophore (e.g., heterocyclic compounds,[9,10] 9-aminoacridine,[11,12] 9-anilinoacridine,[13] 4-anilinoquinoline,[14,15] anthraquinone,[16] and cyclopentanthraquinone[17]) as a DNA affinic carrier. For example, N-mustard targeted by intercalating carriers such as acridine (**1**, Chart 1), and anthraquinones (**2**) can achieve 10- to 100-fold improvement in potency and improve antileukemic activities compared with their corresponding untargeted mustards of similar chemical reactivity,[10,12,16,18] showing altered patterns of DNA alkylation.[6] Most of the recent works, however, have utilized DNA minor groove binders (such as distamycin A and related analogues) as the carriers to offer higher regio- and sequence selectivity and to minimize the limitations of nitrogen mustards.[19–26] In most cases, the DNA-directed alkylating agents have higher cytotoxicity and potency than their corresponding untargeted alkylating agents. Nevertheless, only a limited number of compounds (e.g., tallimustin[27]) were found to have potential clinical application.

Fan et al.[13] synthesized aniline mustard analogues of the DNA-intercalating agent *m*-AMSA (i.e., compounds **3** and **4**) by linking a N-mustard chain to the acridine chromophore and the anilino ring, respectively. At the optimal dose, these analogues exhibited smaller percentage increase in lifespan (%ILS) than the parent *m*-AMSA (**5**) in mice bearing murine leukemia p388.[13] The 4-linked analogues (**3**) showed slightly higher in vivo antileukemic activity than their corresponding 1′-linked analogues (**4**), suggesting that cytotoxic potencies of these compounds may depend on the precise location of the N-mustard moiety on acridine. The decrease in effectiveness of compounds **3** and **4** is probably due to the long linker between the alkylating N-mustard residue and the anilino ring or the acridine chromophore, leading to alternation of the binding site(s) on the DNA and/or drug/topoisomerase II (Topo II) interactions. Taking these into consideration, it may be possible to optimize the cytotoxic activity of these DNA-directed alkylating agents by designing a drug that exhibits both cytotoxic and molecular processes. For example, to enhance the interaction of 9-anilinoacridine with Topo II and/or DNA, one can introduce an alkylating N-mustard residue to the anilino and/or the acridine chromophore with a short spacer between the N-mustard residue and the 9-anilinoacridine core or alter the position of the alkylating moiety on the anilino ring.

Our recent research on the development of new potential antitumor agents demonstrated that 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA·HCl, **6**), a potent Topo II inhibitor, was more cytotoxic than *m*-AMSA (**5**) both in vitro and in vivo.[28–30] Presumably, AHMA exhibits its antitumor activity by stabilizing the DNA–drug–Topo II ternary complex, the cleavable complex,[31] which is highly effective in killing tumor cells. The acridine chromophore of these compounds is generally proposed to be the DNA-binding domain, while the

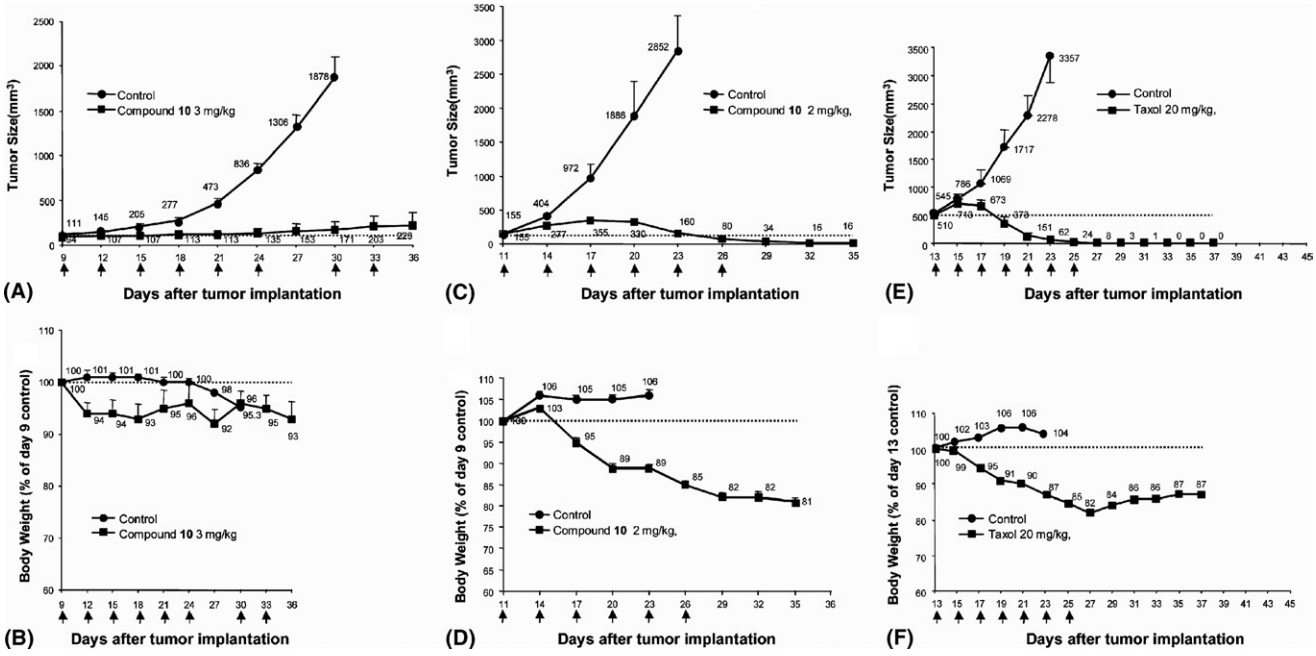


Figure 1. Therapeutic effect of compound **10** (3 mg/kg, Q3D × 8, iv infusion) against human ovarian adenocarcinoma xenograft SK-OV-3 (A) and the body weight changes during treatment as indicated by arrows (B). Therapeutic effect of compound **10** (2 mg/kg, Q3D × 6, iv infusion) and Taxol (20 mg/kg, Q3D × 8, 6 h) (C and E), respectively. The corresponding body weight changes are shown in D and F, respectively.

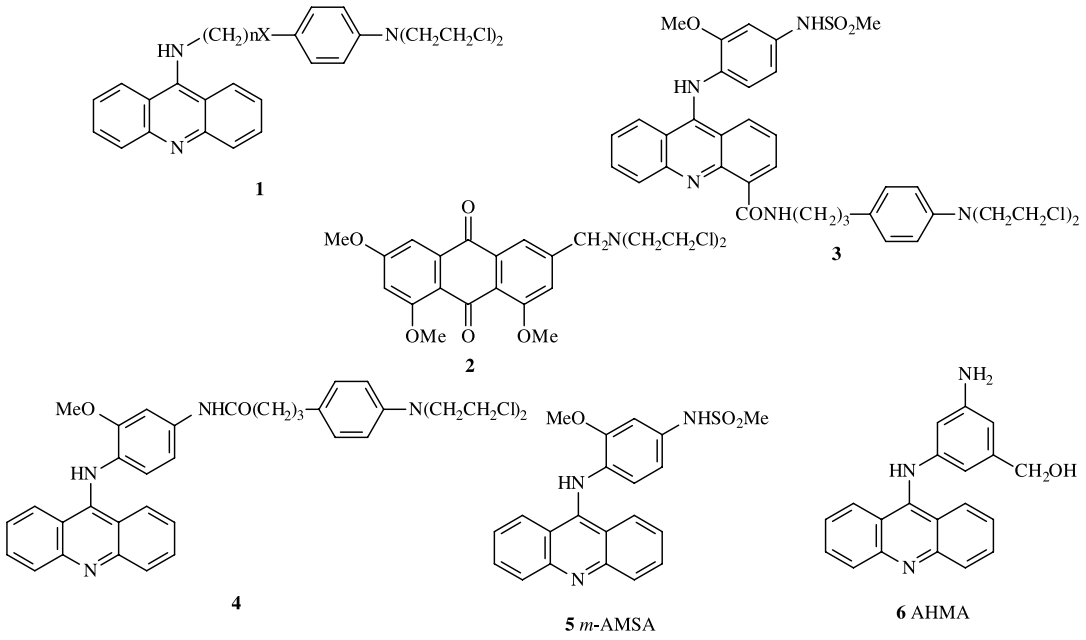


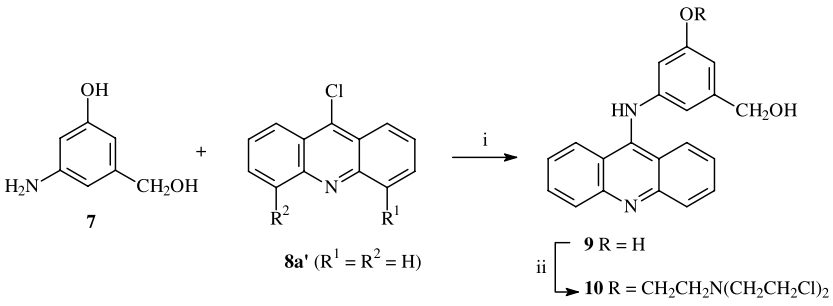
Chart 1. Structures of N-Mustard analogues and AHMA.

anilino moiety is the enzyme-binding domain in these ternary complexes. In spite of this proposed mechanism of action for 9-anilinoacridines, there is a lack of complete understanding of the ternary complex formation.

In view of the intriguing AHMA structure, one can envisage that AHMA may have better interactions with both Topo II and DNA, since AHMA has superior antitumor effects over *m*-AMSA. Therefore, we designed and synthesized AHMA analogues bearing an alkylating N-mustard residue, which was linked to the anilino ring with a short spacer. To introduce a N-mustard alkylating moiety to AHMA, we synthesized a bioisostere of AHMA 3-(9-acridinylamino)-5-hydroxymethylphenol (**9**). Compound **9** was then converted into 9-anilinoacridine bearing a N-mustard moiety (compound **10**) by treating it with tris(2-chloroethyl)amine. We found that compound **10** was approximately 100-fold more cytotoxic than AHMA in inhibiting the cell growth of human lymphoblastic leukemia cells (CCRF–CEM) in culture.[32] This impressive result prompted us to synthesize a series of 9-anilinoacridines bearing a N-mustard, in which the N-mustard was linked to the C-3′ or C-4′ of the anilino ring with a short spacer, such as *O*-ethylene ($O–C_2$), *O*-butylene ($O–C_4$), and methylene (C_1) units. These new target agents allowed us to study the effects of the substituted position of the N-mustard on the anilino ring and the role of spacer length on their antitumor activity. The results revealed that all compounds described in this paper exhibited significant antitumor effects. Here, we described the synthesis, antitumor efficacy and the effects on Topo II-mediated DNA cleavage of the new target compounds.

2. Chemistry

The 9-anilinoacridines bearing an alkylating N-mustard on the anilino ring with an $O–C_2$ spacer were synthesized by directly introducing a N-mustard chain to the OH function of 3-(9-acridinylamino)phenol derivatives (Scheme 1, method 1). The known 3-amino-5-hydroxybenzyl alcohol (**7**) was condensed with 9-chloroacridine (**8a′**) to give 3-(9-acridinylamino)phenol derivative (**9**), which was then treated with 0.2 N potassium hydroxide in methanol to form the solid potassium salt. The salt was then treated with tris(2-chloroethyl)amine in DMF in the presence of K_2CO_3 and KF to produce a



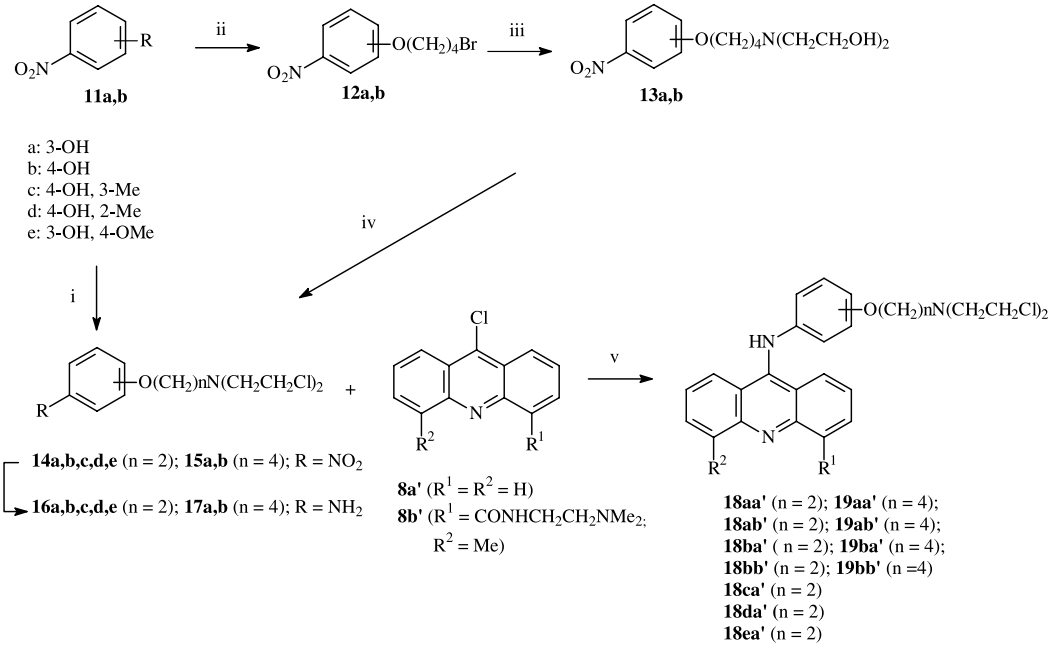
Scheme 1. Reagents and conditions: (i) $CHCl_3$/EtOH/concd HCl (catalytic amount); (ii) K_2CO_3/KF/DMF, 35 °C.

moderate yield of compound **10**. Alternatively, compounds containing an O–C_2 or O–C_4 spacer between the anilino ring and the N-mustard moiety located at the C-3′ or C-4′ position of the anilino ring were synthesized as shown in Scheme 2. Reacting nitro substituted phenol derivatives (**11a–e**) with tris(2-chloroethyl)amine hydrochloride in DMF in the presence of K_2CO_3 and KF in refluxing acetone afforded bis(2-chloroethyl)-[2-(3- or 4-nitrophenoxy)]ethylamines (**14a–e**), which were then reduced ($SnCl_4$/concd HCl) to the corresponding aniline derivatives **16a–e** in low yield. The unstable crude aniline intermediates were used directly without purification for the condensation with the requisite 9-chloroacridines (**8a′**,**b′**) and the desired 9-anilinoacridine derivatives (**18aa′**,**ab′**,**ba′**,**bb′**,**ca′**,**da′**, and **18ea′**) bearing an O–C_2 spacer between the anilino ring and the N-mustard residue were smoothly obtained in moderate yield. Another synthetic route to construct compounds bearing an O–C_4 spacer between the anilino ring and N-mustard was developed (Scheme 2). Thus, treatment of nitrophenols (**11a**,**b**) with excess of 1,4-dibromobutane under similar conditions (K_2CO_3/KF/Me_2CO) gave 1-(4-bromobutoxy)-3- or 4-nitrobenzenes (**12a**,**b**), which were further reacted with diethanolamine to afford bis(2-hydroxyethyl)-[4-(3- or 4-nitrophenoxy)butyl]amines (**13a**,**b**). Prolongation of the reaction of **13a**,**b** with methanesulfonyl chloride in the presence of triethylamine (2–3 days) led to the formation of bis(2-chloroethyl)-[4-(3- or 4-nitrophenoxy)butyl]amines (**15a**,**b**). Apparently, the chlorine anion generated from methanesulfonyl chloride attacked the *O*-methanesulfonyl function of the intermediate to furnish the desired bis(2-chloroethyl) substituted products. The nitro substituted derivatives **15a**,**b** were further reduced ($SnCl_4$/concd HCl) to the corresponding aniline derivatives **17a**,**b**, which were then condensed with 9-chloroacridines (**8a′**,**b′**) to afford the desired 9-anilinoacridines (**19aa′**,**ab′**,**ba′**, and **19bb′**). The N-mustard derivatives of 9-anilinoacridine bearing a C_1 spacer were prepared starting from 3- or 4-nitrobenzyl chloride (**20a** and **20b**, respectively) by the reaction with diethanolamine (Scheme 3). The products (**21a**,**b**) were further converted into the bis(2-chloroethyl)-3- or 4-aminobenzylamines (**23a**,**b**) by treating them with methanesulfonyl chloride followed with reduction as described previously. Similarly, condensation of the crude anilines (**23a**,**b**) with 9-chloroacridines (**8a′**,**b′**) afforded the desired 9-anilinoacridine derivatives (**24aa′**,**ab′**,**ba′**, and **24bb′**).

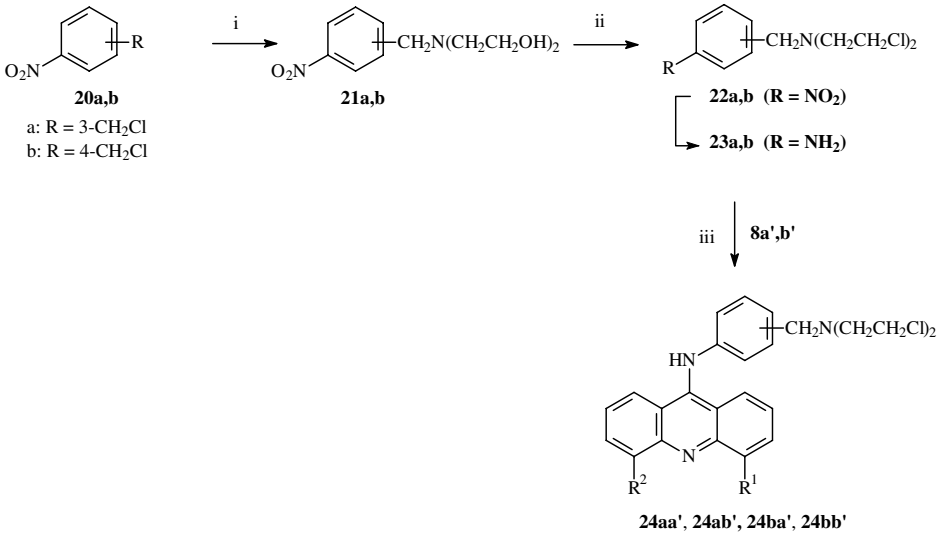
3. Biological results and discussion

3.1. In vitro cytotoxicity

The cytotoxicity of the 9-anilinoacridines bearing an alkylating N-mustard residue on the anilino ring against human lymphoblastic leukemic cells (CCRF–CEM) is shown in Table 1. AHMA (**6**) was used for comparison. This clearly demonstrated that all of the new compounds were significantly more potent than AHMA (**6**). Compound **10** carrying a CH_2OH function with an IC_{50} value of 0.007 μM was about 100-fold more cytotoxic than AHMA (IC_{50} = 0.753 μM), while, compound **18aa′** lacking the CH_2OH substituent was approximately 14-fold less active that **10**. The result, similar to previous observations, confirmed that the CH_2OH function played an important role in the cytotoxicity of AHMA derivatives.[29] In general, compounds with a O–C_2 spacer between the anilino ring and the N-mustard residue were more cytotoxic than their corresponding compounds with a O–C_4 linker located at either C-3′ or C-4′. With one exception, **19bb′** was more

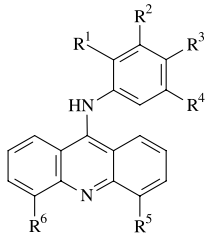


Scheme 2. Reagents and conditions: (i) Tris(2-chloroethyl)amine hydrochloride/K_2CO_3/KF/acetone, reflux; (ii) $Br(CH_2)_4Br$/K_2CO_3/acetone, reflux; (iii) $NH(CH_2CH_2OH)_2$/DMF, 110 °C; (iv) $MeSO_2Cl/Et_3N$, 0 °C to rt, 2 days; (iv) $CHCl_3$/EtOH/concd HCl (catalytic amount).



Scheme 3. Reagents and conditions: (i) $NH(CH_2CH_2OH)_2$, 120 °C; (ii) $MeSO_2Cl/Et_3N$, 0 °C to rt, 2 days; (iii) $CHCl_3$/EtOH/concd HCl (catalytic amount).

Table 1. The in vitro cytotoxicity of N-mustard derivatives of 9-anilinoacridine against human lymphoblastic leukemic cells (CCRF–CEM)



Compound	R^1	R^2	R^3	R^4	R^5	R^6	IC_{50} (μM)
AHMA·HCl **6**	H	H	H	CH_2OH	H	H	0.753
10	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	CH_2OH	H	H	0.0070
18aa′	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	H	H	H	0.095
19aa′	H	$O(CH_2)_4N(CH_2CH_2Cl)_2$	H	H	H	H	0.4555
18ba′	H	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	H	H	0.0200
19ba′	H	H	$O(CH_2)_4N(CH_2CH_2Cl)_2$	H	H	H	0.0610
18ab′	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	H	$CONH(CH_2)_2NMe_2$	Me	0.0030
19ab′	H	$O(CH_2)_4N(CH_2CH_2Cl)_2$	H	H	$CONH(CH_2)_2NMe_2$	Me	0.0235
18bb′	H	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	$CONH(CH_2)_2NMe_2$	Me	0.7730
19bb′	H	H	$O(CH_2)_4N(CH_2CH_2Cl)_2$	H	$CONH(CH_2)_2NMe_2$	Me	0.0230
18ca′	H	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	Me	H	H	0.0263
18da′	Me	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	H	H	H	0.0388
18ea′	H	$O(CH_2)_2N(CH_2CH_2Cl)_2$	OMe	H	H	H	0.0026
24aa′	H	$CH_2N(CH_2CH_2Cl)_2$	H	H	H	H	0.0074
24ba′	H	H	$CH_2N(CH_2CH_2Cl)_2$	H	H	H	0.0300
24ab′	H	$CH_2N(CH_2CH_2Cl)_2$	H	H	$CONH(CH_2)_2NMe_2$	Me	0.0017
24bb′	H	H	$CH_2N(CH_2CH_2Cl)_2$	H	$CONH(CH_2)_2NMe_2$	Me	0.0081

potent than the corresponding **18bb′**. We also found that the substituent(s) on the anilino or acridine ring affected the cytotoxicity of the target compounds: (1) addition of an OMe group to C-4′ of **18ba′** resulted in increased potency (**18ba′** vs **18ea′**); (2) introduction of a Me group to the anilino ring did not affect the cytotoxicity of the parent **18aa′** (**18ca′** or **18da′** vs **18aa′**); (3) addition of a dimethyaminoethylcarboxamido and a Me function to the C4 and C5 of the acridine chromophore further increased the cytotoxicity of the corresponding parent compound with an exception of compound **18bb′** (**18aa′** vs **18ab′**; **18ba′** vs **18bb′**; **19aa′** vs **19ab′**). In the series of compounds without substituents on the acridine chromophore (i.e., **18aa′**, **19aa′**, **18ba′**, and **19ba′**), compounds with alkylating chain at C-4′ were about 8-fold more cytotoxic than the corresponding C-3′-substituted agents. However, the position of the N-mustard residue did not affect the potency of those compounds having substituents on the acridine chromophore. The low cytotoxicity of compound **18bb′** was probably due to its instability in the presence of moisture or water. In contrast to the above observations, in the series of

compounds with a C_1 spacer between the anilino ring and the N-mustard residue, compound **24aa′** (alkylating chain substituted at C-3′) was about 4-fold more potent than the corresponding **24ba′** (C-4′ substitution). Similarly, the substituents (dimethyaminoethylcarboxamido and methyl functions) on the acridine chromophore enhanced the cytotoxicity (3- to 4-fold) of compounds in this series (**24aa′** vs **24ab′** and **24ba′** vs **24bb′**). The most potent compounds in the current studies were **18ab′** and **24ab′** with an IC_{50} value of 0.0028 and 0.0017 μM, respectively.

Table 2 shows the growth inhibition of human lymphoblastic leukemic cells (CCRF–CEM) and its drug-resistant sublines (resistant to vinblastine and Taxol, CCRF–CEM/VBL and CCRF–CEM/Taxol, respectively) by **10**, **18ba′**,**ca′**,**da′**,**ea′**, **24aa′**,**ba′**,**ab′**, and **24bb′**. The results demonstrated that these compounds did not develop cross-resistance to either vinblastine or Taxol. It suggested that the newly synthesized N-mustard derivatives were neither a good substrate of *p*-glycoprotein nor mutated tubulin. It is notable to show that there was no change in the potency when **10** was washed away after incubation for 3 h. Whereas, in the same experiment, the cytotoxicity of AHMA, Taxol, and vinblastine reduced potency by 12-, 205-, and 12-fold, respectively, suggesting covalent binding to the target (probably bind to DNA, Topo II or/and other cell components) in tumor cells by **10**.

The targeting specificity of compound **10** and AHMA (**6**) against topoisomerase I and topoisomerase II was evaluated using three pairs of drug-resistant cell lines, which are known to express reduced levels of topoisomerases. HL-60/MX2 is a topoisomerase II-deficient subline of HL-60 while P388/CPT45 is a topoisomerase I-deficient subline of P388. U937/CR is a camptothecin-resistant subline of U937 with altered topoisomerase I activity. As shown in Table 3, compound **10** exhibited the same in vitro cytotoxicity against the topoisomerase-deficient cells compared to their wild type cells, suggesting that neither topoisomerase I nor topoisomerase II is the primary cytotoxic target of compound **10**. As positive controls, both AHMA·HCl (**6**) and VP-16 were shown to be less cytotoxic against topoisomerase II-deficient HL-60/MX2 cells as compared to their wild type HL-60 cells, consistent with the fact that both are known topoisomerase II-targeting anticancer drugs. Topotecan, a topoisomerase I-specific drug, was also used as a negative control. As shown in Table 3,

Table 2. The cytotoxicity of N-mustard derivatives against human lymphoblastic leukemic cells (CCRF–CEM) and its drug-resistant sublines (CCRF–CEM/VBL and CCRF–CEM/Taxol) and human tumor cell growth in vitro[a]

Compound	IC_{50} (μM)					
	Lymphoblastic leukemic			Solid tumors		
	CCRF–CEM	CCRF–CEM/VBL	CCRF–CEM/Taxol	A549	HCT-116	MX-1
AHMA·HCl (**6**)	0.753	1.60 [2.1×]	0.600 [0.8×]	0.0470 [0.55][b]	ND[c]	0.0035
10	0.0070	0.0075 [1.1×]	0.0340 [4.9×]	0.0056 [0.0050][b]	0.0055	0.0035
18ba′	0.020	0.0665 [4.1×]	0.061 [3.7×]	ND	ND	ND
18ca′	0.0263	0.137 [5.2×]	0.175.6 [6.7×]	0.0769	0.071	0.0344
18da′	0.0388	0.114 [2.9×]	0.147 [3.8×]	0.0900	0.0694	0.0350
18ea′	0.0026	0.00165 [6.3×]	0.0222 [8.5×]	0.0045	0.0068	0.0046
24aa′	0.0056	0.0306 [5.5×]	0.0151 [2.7×]	0.0071	0.0062	0.0053
24ba′	0.0184	0.0665 [4.1×]	0.0610 [3.7×]	0.0209	0.0369	0.0134
24ab′	0.0012	0.0013 [1.1×]	0.0012 [1.0×]	0.0017	0.0032	0.0017
24bb′	0.0076	0.022 [2.9×]	0.018 [2.4×]	0.0129	0.0165	0.0071
Taxol	0.0015	1.62 [1080×]	0.143 [95.3×]	0.0019 [0.390][b]	0.0013	ND
Vinblastine	0.0012	0.540 [450×]	0.029 [24.2×]	0.0081 [0.095][b]	0.0014	ND

[a] XTT assays were used for leukemia cells and SRB assays were used for solid tumor cells. Incubation was 72 h, as described previously.[42] Numbers in the brackets are folds of resistance of the resistant cells when compared with the IC_{50}'s of the CCRF–CEM parent cells.

[b] Incubation for 3 h, washed, and then incubated for a total of 72 h. Washing did not affect BO-742 (**10**) efficacy, whereas efficacy for AHMA, Taxol, and vinblastine were reduced 11.7-, 205-, and 11.7-folds, respectively, due to washing.

[c] ND: not determined.

Table 3. In vitro cytotoxicity of compound **10** and AHMA·HCl (**6**) against various topoisomerase-deficient drug-resistant cell lines[a]

Compound	IC_{50} (μM)[b]					
	HL-60	HL-60/MX2	P388	P388/CPT45	U937	U937/CR
10	0.02	0.02 [1×][c]	0.02	0.02 [1×]	0.03	0.03 [1×]
AHMA·HCl (**6**)	0.2	1.0 [5×]	0.02	0.003 [0.15×]	0.3	0.05 [0.17×]
VP-16	0.06	2.0 [33.3×]	0.02	0.002 [0.1×]	0.05	0.04 [0.8×]
Topotecan	0.03	0.03 [1.0×]	0.03	13 [433×]	0.03	0.03 [1.0×]

[a] HL-60/MX2 cells, a subline of HL-60 selected for resistance to the Topo II drug mitoxantrone, express a lower level of Topo IIα and an undetectable level of Topo IIβ. P388/CPT45 cells and U937/CR cells were camptothecin-resistant variants of P388 and U937, respectively.

[b] Cell growth inhibition IC_{50}'s were determined by a 4 day MTT assay.

[c] Numbers in the brackets are folds of resistance of the resistant cells when compared with the IC_{50}'s of the corresponding parent cells.

topotecan exhibited the same in vitro cytotoxicity against topoisomerase II-deficient HL-60/MX2 cells as compared to their wild type HL-60 cells. As expected, topotecan was less cytotoxic against topoisomerase I-deficient P388/CPT45 cells and U937/CR cells as compared to their respective wild type cells. Taken together, these results suggest that the main mechanism of cytotoxicity of compound **10** is not through its inhibitory effect on topoisomerases.

3.2. In vivo therapeutic activity

Compound **10** and selected derivatives were subjected to comparative in vivo antitumor studies with derivatives of AHMA. Nude mice ($n = 4$) bearing human ovarian carcinoma (SK-OV-3) xenograft were treated with **10** via intravenous infusion for 6 h, Q3D × 8, at the doses of 3.0 mg/kg (on day 9, 12, 15, 18, 21, 24, 30, and 33) upon subcutaneous tumor cell implantation. The study revealed that there was a 96% reduction in the tumor volume with only about 5% of body weight changes (Table 4 and Fig. 1A and B). Table 4 also revealed that the therapeutic efficacy of compound **10** [maximal dose: 2 mg/kg (Q3D × 7); intravenous injection] on nude mice ($n = 3$) bearing human breast carcinoma MX-1 xenograft resulted in complete remission in two out of three mice (Fig. 1C and D). It is worth noting that at the optimal dose, compound **10** attained complete tumor remissions at one tenth of the optimal therapeutic dose of paclitaxel (Taxol®), which is one of the most important agents currently used in clinics, in the MX-1 xenograft model (Fig. 1E and F). Similarly, compound **10** attained 81% suppression of tumor in nude mice bearing human colon carcinoma HCT-116 at 5 mg/kg (Q3D × 5, iv infusion, 6 h). In another experiment, the therapeutic effects and toxicity of compound **10** in nude mice bearing human T-cell acute lymphoblastic leukemic CCRF–CEM xenografts were studied. Table 4 reveals that at 2 mg/kg (Q3D × 6, iv) dose, there was 81% reduction in average tumor size. Based on these studies, compound **10** appeared to be more effective in treating human breast tumor cells than human T-cell lymphoblastic leukemic cells.

However, the therapeutic efficacies of compounds **24aa′**,**ab′**,**ba′**, and **24bb′** were shown to be less potent than that of **10** in the same experimental model (Table 5). About 80% of tumor suppression was observed when mice ($n = 2$) were treated with **24aa′** and **24ab′** at the dose of 5 mg/kg (Q4D × 4), and 5 mg/kg (Q3D × 3) by intravenous injection, respectively. The low potency of **24aa′**,**ab′**,**ba′**, and **24bb′** was probably due to rather the production of inactive bis(2-hydroxyethyl)amine products in vivo system via easy hydrolysis. We have also compared the therapeutic effects of the N-mustard derivatives with that of AHMA derivatives in nude mice bearing MX-1 xenograft (Table 6). This revealed that 68%, 84%, and 81% of average tumor volume was reduced on day 18 when mice were treated with AHMA hydrochloride (**6**·HCl), AHMA–ethylcarbamate (**25**), and AHMA–*tert*-butylcarbamate (**26**)[28] at the dose of 15, 12, and 12 mg/kg (Q2D × 5, intravenous injection), respectively. This clearly demonstrated that **10** was significantly more therapeutically effective than AHMA analogues.

3.3. Topo II-mediated DNA relaxation and DNA cleavage

Comparison of Topo II-mediated relaxation of pRYG–DNA induced by VP-16, compound **10** and AHMA–*tert*-butylcarbamate revealed that these agents inhibited DNA relaxation (Fig. 2). However, compound **10** bind tightly to DNA indicating that **10** might covalently bind to DNA as indicated by retardation in gel mobility and by resistant to washing when A549 tumor cells were treated with this agent. As shown in Figure 3, treatment of ^{32}P-end labeled linear DNA with compound **10**

Table 4. Therapeutic effects of compound **10** in nude mice bearing human xenografts

Tumor used[a]	Dose (mg/kg)	Route and schedule	Maximal body weight[b] (%)	Maximal tumor suppression (%)	Complete tumor disappearance	Toxicity death
CCRF–CEM	2	iv injection, Q3D × 6	28	80.6	0/3	0/3
HCT-116	5	iv infusion, 6 h, Q3D × 5	24	81.5	0/4	0/4
SK-OV-3	3	iv infusion, 6 h, Q3D × 8	8	96.0	0/4	0/4
MX-1	2	iv injection, Q3D × 7	19	>99.1	2/3	0/3

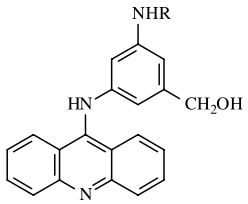
[a] Human tumor tissue 50 mg/nude mouse was implanted subcutaneously on day 0. The dates of starting the treatments were: CCRF–CEM (T-cell acute lymphoblastic leukemia), day 10; HCT-116 (colon carcinoma), day 12; SK-OV-3 (ovarian adenocarcinoma), day 9; and MX-1 (breast carcinoma), day 11.
[b] Body weight = total body weight − tumor weight.

Table 5. Therapeutic effects and toxicity of N-mustard derivatives of 9-anilinoacridine in nude mice bearing human mammary carcinoma (MX-1) xenografts[a]

Compound	Dose (mg/kg)	Schedule	Maximal body weight[b] (%)	Maximal tumor suppression (%)	Tumor free	Death
24aa′	5	Q4D × 3	29	82	0/2	0/2
24ba′	5	Q4D × 4	11	64.8	0/2	0/2
24ab′	1.5	Q3D × 3	29	80	0/2	0/2
24bb′	1.5	Q4D × 4	10	27	0/4	0/4

[a] MX-1 tissue 50 mg was implanted subcutaneously on day 0. Treatment (iv injection) began on day 11 when tumor size were 80–120 mm^3.
[b] Body weight = total body weight − tumor weight.

Table 6. Therapeutic effects of AHMA derivatives in nude mice bearing MX-1 xenograft[a]



Compound	R	Dose (mg/kg)[b] iv, Q2D × 5	Average weight change (gm)				Average tumor volume (*T*/*C*)			*N*	Died of toxicity
			Day 8	Day 14	Day 16	Day 18	Day 14	Day 16	Day 18		
Control			29.6	+0.6	+1.5	+2.7	1.00	1.00	1.00	5	0
6·HCl	H	5	27.6	−0.2	+0.4	+0.5	1.08	0.89	0.82	5	0
		10	15.3	+0.4	+1.0	+2.3	1.12	0.95	0.85	5	0
		15	28.3	+0.0	+0.2	+0.0	0.56	0.42	0.33	4	0
25	COOEt	4	27.1	+0.0	−0.1	+0.9	1.12	0.98	0.88	5	0
		8	28.2	−0.5	−1.1	−0.7	0.91	0.72	0.71	5	0
		12	25.6	−0.2	−0.7	−1.8	0.34	0.26	0.16	3	0
26	$COOCMe_3$	4	27.9	+0.6	+1.0	+2.0	1.06	0.90	1.00	5	0
		8	25.3	+0.4	+1.0	+2.3	1.03	1.00	0.99	4	0
		12	27.6	−0.5	−1.2	−1.1	0.24	0.22	0.19	4	0

[a] MX-1 tissue 50 μg was implanted subcutaneously in mice on day 0. Every other day iv treatments were given on day 8, 10, 12, 14, and 16. The average tumor volume of control group on day 14, 16, and 18 were 179 ± 15, 299 ± 39, and 492 ± 49 mm^3 (mean ± SEM), respectively.
[b] Vehicle used was 20 μL DMSO + 180 μL Saline.

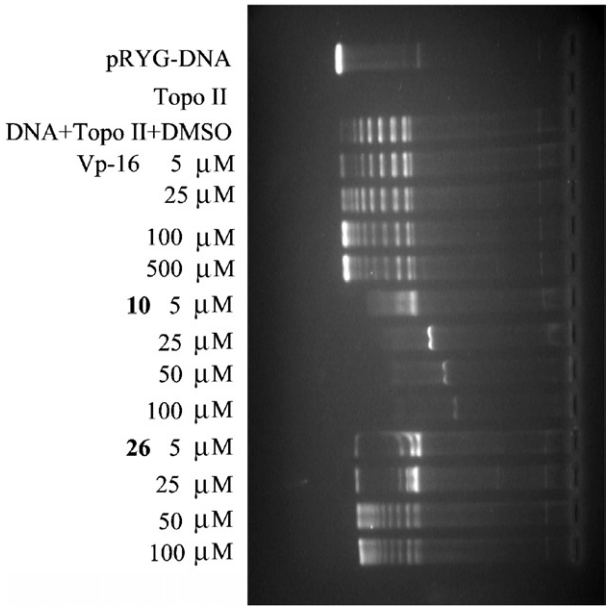


Figure 2. Comparison of Topo II-mediated relaxation of pRYG–DNA induced by VP-16, compound **10** and AHMA–*tert*-butylcarbamate (**26**). These agents inhibited DNA relaxation. However, compound **10** bind tightly to DNA.

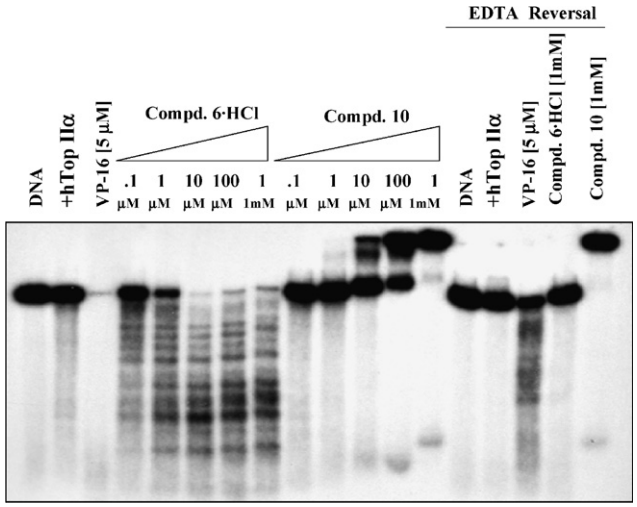


Figure 3. Topoisomerase II-mediated DNA cleavage by AHMA·HCl (**6**), compound **10** and VP-16.

resulted in reduced gel mobility consistent with the formation of a cross-linked DNA dimer, suggesting that compound **10** is a DNA cross-linking agent. The result shown in Figure 3 also demonstrated that compound **10**, unlike AHMA·HCl (**6**), did not induce Topo II-mediated DNA cleavage. These results together with the cytotoxicity results suggest that compound **10**, unlike AHMA, may exert its cytotoxicity primarily through its DNA cross-linking activity.

4. Conclusions

The alkylating N-mustard derivatives exhibit potent antitumor activity and have been applied for cancer chemotherapy. However, these agents have a number of drawbacks due to their high reactivity, resulting in interacting with other cell components and, hence, having less diversion of active drug. Additionally, the majority of N-mustard derivatives are kinetically unstable, leading to the formation of mono-alkylation. Such mono-adducts are considered to be genotoxic rather than cytotoxic. Recently, numerous DNA directed N-mustard derivatives were synthesized for potential therapeutic application. Although, most of these agents

have improvements in potency compared with the corresponding untargeted mustards, only limited compounds were found to have clinical application potential. Most DNA directed N-mustard derivatives possess sequence-specific binding to DNA. However, the DNA sequence-specific binding of these agents, generally, have no direct correlation with their cytotoxicity. This could be due to the loss of their Topo II inhibiting properties. It clearly demonstrates that DNA binding and Topo II inhibition (either caused by Topo II poison or topoisomerase catalytic inhibition) may be viewed as two distinct molecular processes that contribute separately to cytotoxic activity. Solo DNA-alkylation with less or lacking of Topo II inhibitory effect may discriminate the antitumor efficacy of the drug.[13]

The present studies resulted in finding potent antitumor N-mustard derivatives of 9-anilinoacridine in which the alkylating N-mustard residue is linked to the anilino ring with a short spacer (O–C_2, O–C_4, and C_1 unit). We demonstrated that compound **10** exhibits significant cytotoxicity in inhibiting human lymphoblastic leukemia cells (CCRF–CEM) and its drug-resistant sublines (resistant to vinblastine and Taxol, CCRF–CEM/VBL and CCRF–CEM/Taxol, respectively). The efficacious in vivo therapeutic effect of this agent suggested that other compounds reported herein might also have potent therapeutic activity. The studies on the inhibitory effect against Topo II and the cytotoxicity against Topo II and Topo I mutant cell lines of **10** clearly demonstrated that AHMA is a Topo II drug but not Topo I drug whereas **10** is neither. The results also indicated that DNA is the main target for **10**. The interaction of **10** or other derivatives with DNA doubled strands is currently underway in our laboratories to realize the formation of DNA cross-linking or mono-adduct and their sequence-specific binding.

Studies with **10** in nude mice bearing human ovarian carcinoma (SK-OV-3) xenograft possessed potent antitumor therapeutic effect with little toxicity against human tumor xenografts when the agent was administered by iv infusion. Further in vivo therapeutic efficacies of **10** and other derivatives against human tumor xenografts by iv infusion are currently being investigated.

5. Experimental

Melting points were determined on a Fargo melting point apparatus and are uncorrected. Column chromatography was carried out over silica gel G60 (70–230 mesh, ASTM; Merck). Thin layer chromatography was performed on silica gel G60 F_{254} (Merck) plates with short wave length UV light for visualization. Elemental analyses were done on a Heraeus CHN–O Rapid instrument. ^{1}H NMR spectra were recorded on a Brucker AMX-400 spectrophotometer with Me_4Si as an internal standard. The following are the synthesis of the representative compounds. The analytical data and the yield of other new derivatives are shown in Table 7.

5.1. 3-(Acridin-9-ylamino)-5-hydroxymethylphenol (9)

A solution of 9-chloroacridine (**8a**′, 8.56 g, 40 mmol) in $CHCl_3$ (30 mL) was added dropwise to a solution of 3-amino-5-hydroxymethylphenol[33] (7.03 g, 40 mmol) and 4-methylmorpholine (4.2 mL, 4.05 g, 40 mmol) in EtOH (150 mL) at −5 °C for 2.5 h. The reaction mixture was stirred for an additional 1 h in an ice bath and then concentrated in vacuo to dryness and the residue was crystallized from ethanol to give 4.73 g of **9**. Additional product (4.97 g) was obtained from the mother liquid after chromatography (SiO_2, 6 × 30 cm, eluent $CHCl_3$–MeOH, 10:1 v/v); total 9.7 g (75.6%); mp 201–202 °C; ^{1}H NMR (DMSO-d_6): δ 4.43 (2H, s, CH_2), 5.23 (1H, br s, exchangeable, OH), 6.69 (1H, s, ArH), 6.80 (1H, s, ArH), 6.83 (1H, s, ArH), 7.45 (2H, m, 2 × ArH), 7.99 (2H, m, 2 × ArH), 8.10 (2H, m, 2 × ArH), 8.29 (2H, m, 2 × ArH), 9.88 and 11.43 (each 1H, br s, exchangeable, NH or OH). Anal. ($C_{20}H_{15}N_2O_2$·HCl·0.6H_2O) C, H, N.

5.2. (3-(Acridin-9-ylamino)-5-{2-[bis(2-chloroethyl)-amino]ethoxy}phenyl)methanol (10)

A solution of **9** (2.68 g, 8.46 mmol) in 0.2 N KOH/MeOH (42.3 mL, 8.46 mmol) was stirred at room temperature for 10 min and the precipitated orange potassium salt was collected by filtration and dried. The salt was added to a mixture of tris(2-chloroethyl)amine hydrochloride (3.03 g, 12.6 mmol), dry powdered K_2CO_3 (5.80 g, 42 mmol) and dry KF (0.487 mg, 8.46 mmol) in anhydrous DMF (50 mL). The mixture was then gradually heated at 50 °C for 19 h, cooled and then filtered through a pad of Celite, washed with DMF (10 mL). The combined filtrate and washings were evaporated in vacuo to dryness. The residue was dissolved in EtOAc (150 mL), washed with water (50 mL × 3), dried over Na_2SO_4, and evaporated in vacuo to dryness. The residue was chromatographed on a silica gel column (4 × 17 cm) using $CHCl_3$–MeOH (10:1 v/v). The fractions containing the product were combined and evaporated in vacuo to dryness and the solid residue was recrystallized from acetone–hexane (5:1) to give **10**, 2.04 g (50 %); mp 153–154 °C; ^{1}H NMR (DMSO-d_6): δ 2.93 (6H, br s, 3 × NCH_2), 3.60 (4H, br s, 2 × CH_2Cl), 3.96 (2H, br s, OCH_2), 4.43 (2H, s, CH_2OH), 5.05 (1H, s, OH), 6.13 (1H, m, ArH), 6.28 (1H, m, ArH), 6.82 (1H, s, ArH), 7.00–7.12 (1H, m, ArH), 7.29 (2H, m, ArH), 7.46 (3H, m, 3 × ArH), 7.72 (1H, m, ArH), 8.13 (1H, m, ArH), 8.16 (2H, m, ArH), 10.84 (1H, br s, exchangeable, NH). Anal. ($C_{26}H_{27}Cl_2N_3O_2$) C, H, N.

5.3. Bis(2-chloroethyl)-[2-(3-nitrophenoxy)ethyl]amine (14a)

A mixture of *m*-nitrophenol (4.0 g, 28.8 mmol), tris(2-chloroethyl)amine hydrochloride (8.34 g, 34.5 mmol), KF (1.66 g, 28.8 mmol) and K_2CO_3 (19.84 g, 143.8 mmol) in dry acetone (200 mL) was refluxed for 2 days. After cooling, the reaction mixture was filtered and the solid was washed with acetone. The combined filtrate and washings were evaporated in vacuo to dryness and the residue was dissolved in $CHCl_3$ (200 mL), washed with water (3 × 100 mL), dried over Na_2SO_4, and evaporated in vacuo to dryness. The residue was

Table 7. Analytical data and yield of the new compounds

Compound	Chemical formula[a]	Mp (°C)	Yield (%)	Analysis
9	$C_{20}H_{15}N_2O_2{\cdot}1HCl{\cdot}0.6H_2O$	201–202	75.6	C, H, N
10	$C_{26}H_{27}Cl_2N_3O_2$	153–154	50	C, H, N
12a	$C_{10}H_{12}NO_3{\cdot}0.1H_2O$	Syrup	60	C, H, N
12b	$C_{14}H_{22}BrN_2O_5{\cdot}1/2H_2O$	Syrup	67.7	C, H, N
13a	$C_{14}H_{22}N_2O_5{\cdot}1.8H_2O$	Syrup	79.3	C, H, N
13b	$C_{14}H_{22}BrN_2O_5{\cdot}1/2H_2O$	Syrup	62	C, H, N
14a	$C_{12}H_{16}Cl_2N_2O_3{\cdot}2HCl{\cdot}2H_2O$	119–120	25	C, H, N
14b	$C_{12}H_{16}Cl_2N_2O_3{\cdot}HCl$	188–189	21	C, H, N
14c	$C_{13}H_{18}Cl_2N_2O_3{\cdot}HCl$	158–159	24	C, H, N
14d	$C_{13}H_{18}Cl_2N_2O{\cdot}HCl$	152–153	29	C, H, N
14e	$C_{13}H_{18}Cl_2N_2O_4{\cdot}HCl$	181–182	16	C, H, N
15a	$C_{14}H_{20}Cl_2N_2O_3{\cdot}HCl$	120–121	60	C, H, N
15b	$C_{14}H_{20}Cl_2N_2O_3{\cdot}HCl$	166–167	85	C, H, N
18aa′	$C_{25}H_{25}Cl_2N_3O{\cdot}1.HCl{\cdot}5H_2O$	123–124	36	C, H, N
18ab′	$C_{31}H_{37}N_5O_2Cl_2{\cdot}4HCl{\cdot}3H_2O$	129–130	41	C, H, N
18ba′	$C_{25}H_{25}Cl_2N_3O{\cdot}2HCl{\cdot}0.5H_2O$	225–226	29	C, H, N
18bb′	$C_{31}H_{37}Cl_2N_5O_2{\cdot}5/2HCl{\cdot}4H_2O$	185–186	67	C, H, N
18ca′	$C_{26}H_{27}Cl_2N_3O{\cdot}3/2HCl{\cdot}5/2H_2O$	235–236	50	C, H, N
18da′	$C_{26}H_{27}Cl_2N_3O{\cdot}3/2HCl{\cdot}H_2O$	197–198	55	C, H, N
18ea′	$C_{26}H_{27}Cl_2N_3O_2{\cdot}5/4HCl{\cdot}H_2O$	134–145	58	C, H, N
19aa′	$C_{27}H_{29}Cl_2N_3O{\cdot}2{\cdot}HCl{\cdot}5H_2O$	126–127	30	C, H, N
19ab′	$C_{33}H_{41}Cl_2N_5O_2{\cdot}3HCl{\cdot}7H_2O$	131–132	26	C, H, N
19ba′	$C_{27}H_{29}Cl_2N_3O{\cdot}3HCl{\cdot}0.5H_2O$	109–110	22	C, H, N
19bb′	$C_{33}H_{41}Cl_2N_5O_2{\cdot}1.5HCl{\cdot}4H_2O$	166–167	71	C, H, N
21a	$C_{11}H_{16}N_2O_4$	71–72	58	C, H, N
21b	$C_{11}H_{16}N_2O_4$	74–75	80.5	C, H, N
22a	$C_{11}H_{14}Cl_2N_2O_2{\cdot}HCl$	153–154	89	C, H, N
22b	$C_{11}H_{14}Cl_2N_2O_2{\cdot}HCl$	45–46	73	C, H, N
24aa′	$C_{24}H_{23}Cl_2N_3{\cdot}2HCl{\cdot}0.5H_2O$	207–208	85	C, H, N
24ab′	$C_{30}H_{35}Cl_2N_5O{\cdot}3HCl{\cdot}2H_2O$	198–199	92	C, H, N
24ba′	$C_{24}H_{23}Cl_2N_3{\cdot}2HCl{\cdot}0.5H_2O$	220–223	35	C, H, N
24bb′	$C_{30}H_{35}Cl_2N_5O{\cdot}3HCl{\cdot}2H_2O$	204–205	76.3	C, H, N

[a] Compounds are hygroscopic and contain crystal water.

chromatographed on a silica gel column (5 × 7 cm) using CH_2Cl_2 as the eluent. The fractions containing the desired product were combined and evaporated under reduced pressure to give **14a** as syrup, 2.2 g (25%); mp 119–120 °C (HCl salt, EtOH); ^{1}H NMR ($CDCl_3$): δ, 3.05 (4H, t, J = 6.9 Hz, $2 \times NCH_2$), 3.10 (2H, t, J = 5.5 Hz, NCH_2), 3.57 (4H, t, J = 6.9 Hz, $2 \times CH_2Cl$), 4.12 (2H, t, J = 5.5 Hz, OCH_2), 7.21 (1H, dq, J = 2.5, 8.3 Hz, ArH), 7.41 (1H, t, J = 8.8 Hz, ArH), 7.69 (1H, t, J = 2.5 Hz, ArH), 7.79 (1H, dq, J = 2.5, J = 8.3 Hz, ArH). Anal. ($C_{12}H_{16}Cl_2N_2O_3{\cdot}2HCl{\cdot}2H_2O$) C, H, N.

By following the same procedure as that for **14a**, compounds **14b–e** (Table 7) were synthesized.

5.4. 1-(4-Bromobutoxy)-3-nitrobenzene (12a)

A mixture of *m*-nitrophenol (**11a**, 4.99 g, 35.9 mmol), 1,4-dibromobutane (9.30 g, 43.1 mmol), and K_2CO_3 (7.43 g, 53.9 mmol) in acetone (200 mL) was refluxed for 4 h. After cooling, the reaction mixture was filtered through a pad of Celite and the filter cake was washed with acetone. The combined filtrate and washings were evaporated in vacuo to dryness. The residue was diluted with water (100 mL) and extracted with $CHCl_3$ (6 × 100 mL). The organic extracts were combined, washed successively with 1% NaOH (50 mL) and water (30 mL), dried over Na_2SO_4, and evaporated under reduced pressure to dryness. The residue was chromatographed on a silica gel column (5 × 20 cm) using $CHCl_3$ as an eluent. The fractions containing the desired product were combined, evaporated in vacuo to give **12a**, yield 5.95 g (60%) as syrup; ^{1}H NMR ($CDCl_3$): δ 2.02 (2H, m, CH_2), 2.08 (2H, m, CH_2), 3.50 (2H, t, J = 6.2 Hz, CH_2Br), 4.78 (2H, t, J = 6.2 Hz, OCH_2), 7.22 (1H, dq, J = 2.7, 8.3 Hz, ArH), 7.43 (1H, t, J = 8.1 Hz, ArH), 7.69 (1H, dq, J = 2.7, 8.3 Hz, ArH), 7.89 (1H, m, ArH). Anal. ($C_{10}H_{12}NO_3{\cdot}0.1H_2O$) C, H, N.

By following the same procedure as that for compound **12a**, compound **12b** (Table 7) was prepared.

5.5. 2-{(2-Hydroxyethyl)-[4-(3-nitrophenoxy)butyl]-amino}ethanol (13a)

A mixture of **12a** (5.0 g, 21.7 mmol) and diethanolamine (6.85 g, 65.1 mmol) in DMF was heated at 115 °C with vigorous stirring for 30 min. After cooling, the mixture was concentrated under reduced pressure to remove the solvent. The residue was washed successively with hexane (5 × 50 mL) and ether (2 × 30 mL) and was then dissolved in $CHCl_3$ (200 mL). The $CHCl_3$ solution was washed with water (6 × 80 mL) to remove excess diethanolamine, dried over Na_2SO_4, and evaporated in vacuo to dryness. Compound **13a**, 5.23 g (79.3%), was

obtained as syrup; ^{1}H NMR ($CHCl_3$): δ, 1.67 (2H, m, CH_2), 1.83 (2H, m, CH_2), 2.62 (2H, t, J = 7.2 Hz, NCH_2), 2.67 (4H, t, J = 5.4 Hz, 2 × NCH_2), 3.62 (4H, m, 2 × CH_2OH), 4.04 (2H, t, J = 5.3 Hz, OCH_2), 7.21 (1H, dq, J = 2.7, 8.3 Hz, ArH), 7.41 (1H, t, J = 8.2 Hz, ArH), 7.69 (1H, s, ArH), 7.78 (1H, dq, J = 2.7, 8.3 Hz, ArH). Anal. ($C_{14}H_{22}N_2O_5 \cdot 1.8H_2O$) C, H, N.

By following the same procedure as that for compound **13a**, compounds **13b**, **21a**, and **21b** (Table 7) were synthesized.

5.6. Bis(2-chloroethyl)-[4-(3-nitrophenoxy)butyl]amine (15a)

Methanesulfonyl chloride (5.75 g, 50.3 mmol) was added dropwise to a solution of **13a** (5.0 g, 16.8 mmol) and triethylamine (6.78 g, 67.0 mmol) in dry $CHCl_3$ (25 mL) in an ice bath. The reaction mixture was stirred for 3 days at room temperature and then diluted with $CHCl_3$ (150 mL). The solution was washed successively with cold aqueous solution of 10% $NaHCO_3$ (50 mL) and ice water (100 mL), dried over Na_2SO_4, and evaporated in vacuo to dryness. The residue was recrystallized from EtOH to give pale yellow crystals, 3.4 g (60%); mp 120–121 °C; ^{1}H NMR ($CHCl_3$): δ, 1.97 (2H, m, CH_2), 2.15 (2H, m, CH_2), 3.37 (2H, t, J = 8.2 Hz, NCH_2), 3.57 (4H, s, $2NCH_2$), 4.10 (6H, m, OCH_2 and 2 × CH_2Cl), 7.22 (1H, dq, J = 8.3 Hz, ArH), 7.44 (1H, t, J = 2.7, 8.2 Hz, ArH), 7.77 (1H, s, ArH), 7.84 (1H, dq, J = 2.7, 8.3 Hz, ArH). Anal. ($C_{14}H_{20}Cl_2N_2O_3 \cdot 1.8H_2O$) C, H, N.

By following the same procedure as that for compound **15a**, compounds **15b**, **22a**, and **22b** (Table 7) were synthesized.

5.7. Acridin-9-yl-(3-{2-[bis(2-chloroethyl)amino]ethoxy}-phenyl)amine (18aa′)

A mixture of bis(2-chloroethyl)-[2-(3-nitrophenoxy)-ethyl]amine (**14a**) (307 mg, 1.0 mmol) and $SnCl_2 \cdot 2H_2O$ (675 mg, 3.0 mmol) in concd HCl (4 mL) was stirred at 60 °C for 30 min. The clear solution was poured into ice (25 g) and neutralized slowly with 25% NH_4OH. The mixture was extracted with $CHCl_3$ (4 × 50 mL), dried over Na_2SO_4, and evaporated in vacuo to dryness to give crude 3-bis(2-chloroethyl)aminoethoxyaniline (**16a**), which was dissolved in $CHCl_3$ (20 mL) and added to a solution of 9-chloroacridine (**8a**, 106 mg, 0.5 mmol) in $CHCl_3$ (20 mL) containing two drops of concd HCl in an ice bath. After being stirred at room temperature for 6 h, the mixture was evaporated in vacuo to dryness and the residue was chromatographed on silica gel column (1 × 20 cm) using $CHCl_3$–methanol (10:1 v/v) as the eluant. The main fractions containing the desired product were combined and evaporated in vacuo to dryness. The residue was treated with excess dry HCl in EtOAc (2.5 N) and then evaporated under reduced pressure to dryness. The solid residue was recrystallized from acetone/ethyl acetate to give **18aa′**, 164 mg (36%) as HCl salt; mp 123–124 °C; ^{1}H NMR (DMSO-d_6): δ 2.97 (6H, t, J = 7.1 Hz, 3 × NCH_2), 3.49 (4H, t, J = 7.1 Hz, 2 × CH_2Cl), 3.94 (2H, t, J = 5.4 Hz, OCH_2), 6.51 (2H, m, ArH), 6.56 (1H, m, ArH), 7.17 (1H, m, ArH), 7.22 (2H, br s, ArH), 7.60 (2H, m, ArH), 7.94 (2H, m, ArH), 8.04 (2H, m, ArH), 11.21 (1H, br s, NH). Anal. ($C_{25}H_{25}Cl_2N_3O \cdot HCl \cdot 5H_2O$) C, H, N.

By following the same procedure as that for the synthesis of compound **18aa′**, compounds **18ab′**,**ba′**,**bb′**, **ca′**,**da′**,**ea′**, **19aa′**,**ab′**,**ba′**,**bb′**, **24aa′**,**ab′**,**ba′**, and **24bb′** (Table 7) were prepared.

6. Biological experiments

6.1. Cytotoxicity assays

The effects of the compounds on cell growth were determined in all human tumor cells (i.e., lung adenocarcinoma A549, colon carcinoma HCT-116, breast carcinoma MX-1, and T-cell acute lymphocytic leukemia CCRF–CEM), in a 72 h incubation, by XTT–tetrazolium assay, as described by Scudiero et al.[34] After the addition of phenazine methosulfate-XTT solution at 37 °C for 6 h, absorbance at 450 and 630 nm was detected on a microplate reader (EL 340; Bio-Tek Instruments Inc., Winooski, VT). Six to seven concentrations of each compound were used. The IC_{50} and dose–effect relationships of the compounds for antitumor activity were calculated by a median–effect plot,[35,36] using a computer program on an IBM-PC workstation.[37] The cytotoxicity of compound **10** and AHMA·HCl against a number of drug-resistant cell lines with an altered expression of Topo I or Topo II was measured by the MTT assay as described.[38]

6.2. In vivo studies

Athymic nude mice bearing the nu/nu gene were used for human breast tumor MX-1, human T-cell acute lymphoblastic leukemia CCRF-CEM, human colon carcinoma HCT-116, and human ovarian adenocarcinoma SK-OV-3 xenografts. Outbred Swiss-background mice were obtained from Charies River Breeding Laboratories. Male mice 8 weeks old or older weighing 22 g or more were used for most experiments. Drug was administrated via the tail vein by iv injection. Tumor volumes were assessed by measuring length × width × height (or width) by using a caliper. Vehicle used was 20 μL DMSO in 180 μL saline. All animal studies were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Animals and the protocol approved by the Memorial Sloan-Kettering Cancer Center's Institutional Animal Care and Use Committee.

6.3. Topoisomerase II–DNA relaxation assay

Topo II–DNA relaxation activities were determined by following the procedures described by Liu and Miller[39] and Hirose et al.[40] The reaction mixture (50 μL) containing 10 mM Tris, pH 7.9, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1 mM ATP, 30 μg/mL BSA, 15 μg/mL SV40 DNA, or

pBR322 DNA, and a known amount of the Topo II, were incubated at 30 °C for 15 min. The reaction was stopped by the addition of SDS to 1%, EDTA to 20 mM, and the proteinase K to 400 μg/mL; the mixture was further incubated at 37 °C for 30 min, mixed with dye solution containing 5% sucrose and 0.01% xylene cyanol, and analyzed on a 1% agarose gel using TBE (89 mM Tris–borate, 2 mM EDTA) containing 0.1% SDS.

6.4. Topoisomerase II-mediated DNA cleavage assay

Topo II-mediated DNA cleavages were determined by following the procedures previously described.[41] The reaction mixture (20 μL each) containing 40 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP, 30 μg/mL bovine serum albumin, 20 ng of 3′-end ^{32}P-labeled YEpG DNA, 10 ng of purified hTopo IIα and a test compound was incubated at 37 °C for 30 min. The reactions were terminated by addition of 5 μL of a solution containing 5% SDS and 1 mg/mL proteinase K followed by incubation for an additional 60 min at 37 °C. EDTA reversal was performed by adding EDTA to 20 mM at the end of the first incubation. Following a second incubation at 37 °C for 10 min, reactions were then terminated by SDS and proteinase K as described above. DNA samples were electrophoresed in 1% agarose gel containing 0.5× TPE buffer. Gels were dried onto Whatman 3MM chromatographic paper and autoradiographed at −80 °C using Kodak XAR-5 film.

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Appendix A. C, H, N analysis

Compound	Chemical formula	Anal. Calcd			Found		
		C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
9	$C_{20}H_{15}N_2O_2 \cdot HCl \cdot 0.6H_2O$	65.84	5.07	7.68	65.86	65.86	7.51
10	$C_{26}H_{27}Cl_2N_3O_2$	64.24	5.62	8.66	64.20	5.88	8.28
12a	$C_{10}H_{12}NO_3 \cdot 0.1H_2O$	43.53	4.46	5.07	44.10	4.52	5.09
12b	$C_{14}H_{22}BrN_2O_5 \cdot 1/2H_2O$	54.71	7.21	9.11	54.58	7.50	9.00
13a	$C_{14}H_{22}N_2O_5 \cdot 1.8H_2O$	50.60	7.28	8.43	50.65	7.02	8.18
13b	$C_{14}H_{22}BrN_2O_5 \cdot 1/2H_2O$	54.71	7.21	9.11	54.58	7.50	9.00
14a	$C_{12}H_{16}Cl_2N_2O_3 \cdot 2HCl \cdot 2H_2O$	34.45	5.30	6.69	34.45	5.31	6.53
14b	$C_{12}H_{16}Cl_2N_2O_3 \cdot HCl$	41.94	4.99	8.15	41.78	5.04	8.02
14c	$C_{13}H_{18}Cl_2N_2O_3 \cdot HCl$	43.65	5.35	7.83	43.44	5.38	7.69
14d	$C_{13}H_{18}Cl_2N_2O \cdot HCl$	43.65	5.35	7.83	43.45	5.40	7.73
14e	$C_{13}H_{18}Cl_2N_2O_4 \cdot HCl$	41.78	5.12	7.50	41.69	5.07	7.50
15a	$C_{14}H_{20}Cl_2N_2O_3 \cdot 1.8H_2O$	45.55	6.03	7.58	45.55	5.76	7.50
15b	$C_{14}H_{20}Cl_2N_2O_3 \cdot HCl$	45.07	5.67	7.53	45.25	5.75	7.50
18aa′	$C_{25}H_{25}Cl_2N_3O \cdot HCl \cdot 5H_2O$	51.68	6.25	7.23	51.70	6.30	7.26
18ab′	$C_{31}H_{37}N_5O_2Cl_2 \cdot 4HCl \cdot 3H_2O$	47.60	6.06	8.95	47.60	6.45	8.91
18ba′	$C_{25}H_{25}Cl_2N_3O \cdot 2HCl \cdot 0.5H_2O$	55.98	5.26	7.84	56.06	5.33	7.84
18bb′	$C_{31}H_{37}Cl_2N_5O_2 \cdot 5/2HCl \cdot 4H_2O$	49.86	6.41	9.38	50.21	6.67	9.65
18ca′	$C_{26}H_{27}Cl_2N_3O \cdot 3/2HCl \cdot 5/2H_2O$	53.45	5.78	7.19	53.54	5.88	7.07
18da′	$C_{26}H_{27}Cl_2N_3O \cdot 3/2HCl \cdot H_2O$	57.70	5.68	5.68	58.01	58.01	7.73
18ea′	$C_{26}H_{27}Cl_2N_3O_2 \cdot 5/4HCl \cdot H_2O$	56.98	5.56	7.67	56.76	5.52	7.70
19aa′	$C_{27}H_{29}Cl_2N_3O \cdot 2 \cdot HCl \cdot 5H_2O$	50.24	6.40	6.51	50.61	6.53	6.27
19ab′	$C_{33}H_{41}Cl_2N_5O_2 \cdot 3HCl \cdot 7H_2O$	46.84	6.91	8.28	46.91	6.75	8.22
19ba′	$C_{27}H_{29}Cl_2N_3O \cdot 3HCl \cdot 0.5H_2O$	53.97	5.53	6.99	53.34	5.43	7.03
19bb′	$C_{33}H_{41}Cl_2N_5O_2 \cdot 1.5HCl \cdot 4H_2O$	44.87	6.21	7.92	44.95	6.24	7.84
21a	$C_{11}H_{16}N_2O_4$	54.99	6.71	11.66	55.03	6.72	11.63
21b	$C_{11}H_{16}N_2O_4$	54.99	6.71	11.66	55.14	6.74	11.64
22a	$C_{11}H_{14}Cl_2N_2O_2 \cdot HCl$	42.13	4.82	8.93	41.29	4.82	8.80
22b	$C_{11}H_{14}Cl_2N_2O_2 \cdot HCl$	42.13	4.82	8.93	42.36	5.05	8.78
24aa′	$C_{24}H_{23}Cl_2N_3 \cdot 2HCl \cdot 0.5H_2O$	56.93	5.18	8.30	55.63	5.20	8.29
24ab′	$C_{30}H_{35}Cl_2N_5O \cdot 3HCl \cdot 2H_2O$	51.62	6.07	10.03	51.50	6.20	10.19
24ba′	$C_{24}H_{23}Cl_2N_3 \cdot 2HCl \cdot 0.5H_2O$	56.93	5.18	8.30	56.86	5.31	8.16
24bb′	$C_{30}H_{35}Cl_2N_5O \cdot 3HCl \cdot 2H_2O$	51.62	6.07	10.03	51.39	6.26	9.97

Supplementary data

The ^{1}H NMR spectroscopic data of compounds listed in Table 7. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.03.057.

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