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Synthesis and antitumor activity of 5-(9-acridinylamino)anisidine derivatives

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Abstract—A series of 5-(9-acridinylamino)anisidines were synthesized by condensing methoxy-substituted 1,3-phenylenediamines (**10** and **11**) with 9-chloroacridine derivatives to form 5-(9-acridinylamino)-*m*-anisidines (AMAs, **14a**–**e**) and 5-(9-acridinylamino)-*o*-anisidines (AOAs, **15a**–**e**). 5-(9-Acridinylamino)-*p*-anisidines (APAs, **17a**–**e**) were synthesized by reacting 2-methoxy-5-nitroaniline (**12**) with 9-anilinoacridines, followed by reduction. The cytotoxic inhibition of growth of various human tumor cells in culture, inhibitory effects against topoisomerase II, and DNA interaction of these agents were studied. The structure–activity relationship studies revealed the following degree of potency: AOAs > AMAs > APAs. They also revealed that the newly synthesized derivatives bearing $CONH_2NH_2NMe_2$ and Me substituents at C4 and C5 positions of the acridine chromophore (i.e., AMA **14e**, AOA **15e**, and APA **17e**) exhibited significant cytotoxicity against human tumor cell growth in vitro. AOA (**15e**) was the most potent among these derivatives, which resulted in 60% suppression of tumor volume at a dose of 20 mg/kg (Q2D × 9), intravenous injection on day 26 in nude mice bearing human breast carcinoma MX-1 xenografts.

1. Introduction

Among antitumor DNA-intercalating agents and topoisomerase II (Topo II) inhibitors, 9-anilinoacridines have been extensively investigated. Numerous attempts have been made to design and synthesize compounds with potent antitumor therapeutic efficacy and bioavailability for clinical application by modifying the substituent(s) on the anilino ring and/or acridine chromophore,[1,2] in addition to quantitative structure–activity relationships (QSAR) studies.[3–5] Among these agents, *m*-amsacrine (*m*-AMSA) and its 4-methyl-5-methylcarboxamide derivative (CI-921) were of particular interest in that *m*-AMSA was subjected to clinical investigations for the treatment of acute leukemia,[6,7] and CI-921 was in phase II clinical trials for treatment of solid tumors.[8–11] The results of these studies showed that both agents were too toxic or did not sustain human antitumor efficacy.[12,13]

As reviewed by Gao et al.,[5] the QSAR studies of 9-anilinoacridine congeners have demonstrated that the electronic effect of substituent(s) is associated with the drug's binding site, while hydrophobicity is associated with drug's entrance to the active site and steric effect of the substituent is associated with drug's binding to an active site on a macromolecule. These points were the three consistent critical conclusions drawn from these QSAR studies. However, there is a need to have more systematical investigations using these complicated QSAR studies to develop more desirable agents for potential clinical applications.

Previous studies examining the development of potential anticancer agents, namely 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **1**, Fig. 1) and its derivatives, have demonstrated that AHMA exhibited both potent in vitro and in vivo antitumor efficacies[2] and superior antitumor efficacy when compared to both *m*-AMSA and VP-16 in mice bearing mammary carcinoma.[14] Additionally, the alkylcarbamates of AHMA (AHMA-alkylcarbamates, e.g., **2**) were more effective than their corresponding parent AHMA derivatives, perhaps due to increased hydrophobicity of these

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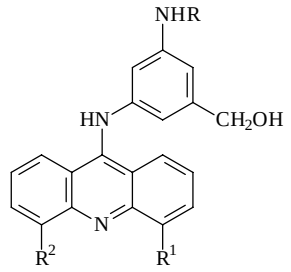
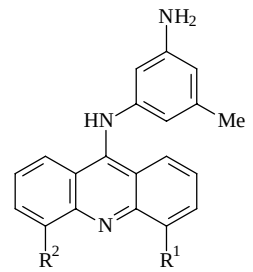
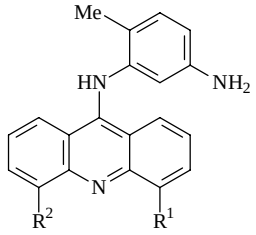


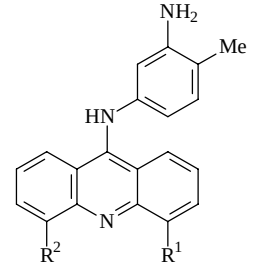
1 R = R^1 = R^2 = H (AHMA)
2 R = COOEt, R^1 = R^2 = H
3 R = H, R^1 = $CONHCH_2CH_2NMe_2$, R^2 = Me,



4 R^1 = R^2 = H (AMT)
5 R^1 = $CONHCH_2CH_2NMe_2$, R^2 = Me,



6 R^1 = R^2 = H (APT)
7 R^1 = $CONHCH_2CH_2NMe_2$, R^2 = Me,



8 R^1 = R^2 = H (AOT)
9 R^1 = $CONHCH_2CH_2NMe_2$, R^2 = Me,

Figure 1.

agents.[15] The structure–activity relationship studies of AHMA derivatives have demonstrated that the CH_2OH functional group on the anilino ring played an important role in their antitumor activity, probably via involvement in drug/DNA binding. More recently, we found that conversion of the CH_2OH group to an ester increases its hydrophobicity and the cytotoxicity of **2** was enhanced further. Replacing the CH_2OH substituent with a Me group at the *meta*-, *para*- or *ortho*-position to the NH_2 group, 5-(9-acridinylamino)-*m*-toluidines (AMTs, **4** and **5**), 5-(9-acridinylamino)-*p*-toluidines (APTs, **6** and **7**), or 5-(9-acridinylamino)-*o*-toluidines (AOTs, **8** and **9**) were formed, respectively, which were more hydrophobic than the parent AHMA.[16] The results showed that compounds **4**, **6**, and **8** were less cytotoxic than AHMA, indicating that either removal of the CH_2OH or replacement of this functional group with Me reduced its cytotoxicity further and weakened its inhibitory effect against Topo II. However, 5-(9-acridinylamino)toluidine analogs (**5**, **7**, and **9**) bearing $CONHCH_2CH_2NMe_2$ and Me functions at C4 and C5 of the acridine chromophore, respectively, were more potent than AHMA (**1**) and AHMA-ethylcarbamate (**2**) or as potent as AHMA derivative **3** depending on the tumor cell line tested. These three agents exhibited potent therapeutic effects in mice bearing human breast carcinoma MX-1 xenografts. Moreover, APTs and AOTs were more potent than AMTs, perhaps due to the inductive effect of the Me function.

The studies indicated that the electronegativity of the anilino ring induced by the Me group may affect the drug/enzyme interaction and consequently the drug's cytotoxicity. For example, the substituents ($CONHCH_2CH_2NMe_2$ and Me functions) on the acridine chromophore may have influenced the drug/DNA interaction and hence altered the cytotoxicity of the 9-anilinoacridines. Earlier reports have demonstrated that adding an electron-donor OMe group on the anilino ring of 9-anilinoacridine increased its antitumor potency.[17] Based on these findings, one can envisage that the cytotoxicity of 5-(9-acridinylamino)toluidines can be enhanced further by replacing the Me function with the elevated electron-donating OMe group to increase the electronegativity of the anilino ring. In this paper, we described the synthesis and biological evaluations of a series of 5-(9-acridinylamino)-*m*-anisidines (AMAs), 5-(9-acridinylamino)-*p*-anisidines (APAs), and 5-(9-acridinylamino)-*o*-anisidines (AOAs). The structure–activity relationship studies concluded that the target AOAs and AMAs were more cytotoxic than the corresponding AOTs and AMTs in inhibiting growth of various human tumor cells in vitro, with the exception of APAs.

2. Chemistry

5-(9-Acridinylamino)-*m*-anisidines (AMAs, **14a**–**e**) were synthesized by the condensation of 3,5-diaminoanisole dihydrochloride (**10**) with the known 9-chloroacridines (**13a**–**e**) in $CHCl_3$/EtOH in the presence of 4-methylmorpholine in an ice/MeOH bath by following a procedure previously developed in our laboratory.[14,16] Under such conditions, good yield of **14a**–**e** was obtained as hydrochloride salts. In a similar manner, the reaction of 2,4-diaminoanisole dihydrochloride (**11**)

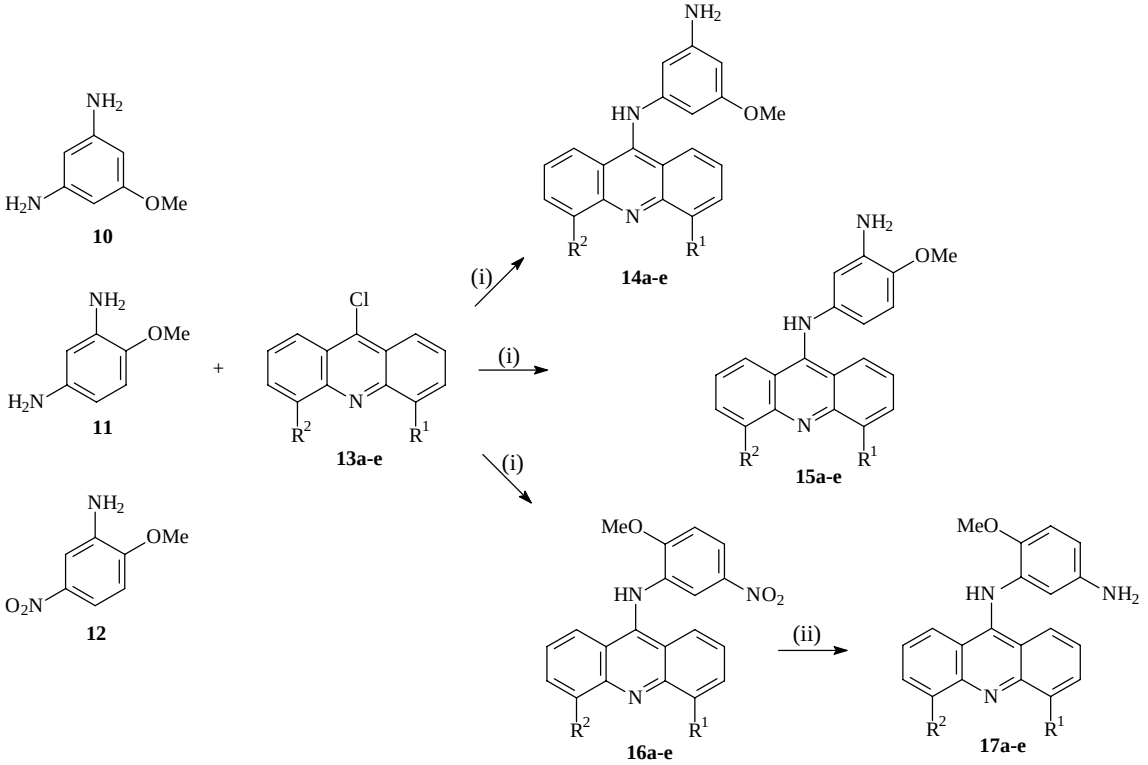
with the requisite 9-chloroacridines (**13a**–**e**) afforded 5-(9-acridinylamino)-*o*-anisidines (AOAs, **15a**–**e**) in good yield. It is interesting to note that the reaction of **11** with **13a**–**e** may afford the isomers AOAs (**15a**–**e**) and/or 5-(9-acridinylamino)-*p*-anisidines (APAs, **17a**–**e**). To identify the structure of **15a**–**e**, we first treated 2-methoxy-5-nitroaniline (**12**) with 9-chloroacridines (**13a**–**e**) in a mixture of $CHCl_3$/EtOH in the presence of a catalytic amount of concd hydrochloric acid. The product, acridin-9-yl-(2-methoxy-5-nitrophenyl)amines (**16a**–**e**), was then converted to APAs (**17a**–**e**) upon catalytic hydrogenation (5% Pd/C, H_2) (see Scheme 1).

The structures of AOAs (**15a**–**e**) and APAs (**17a**–**e**) were determined by the ^{1}H NMR spectroscopy method. The ^{1}H NMR (DMSO-d_6) spectra of the series of AOAs (**15a**–**e**) and APAs (**17a**–**e**) revealed that chemical shifts for their OMe substituent can be clearly distinguished; the signal for protons of the OMe functional group in AOAs (**15a**–**e**) locates appears at a lower field than that of APAs (**17a**–**e**) with δ ~3.84–3.91 and ~3.36–3.45, respectively. While the chemical shifts of OMe groups in AMAs (**15a**–**e**) are about δ ~3.64–3.67. Apparently, the NH_2 function at C4 of 2,4-diaminoanisole (**11**) is more reactive than the one at C2, resulting in the formation of AOAs (**15a**–**e**) as the prominent products. In contrast, our previous study showed that the reaction of 2,4-diaminotoluene with 9-chloroacridines (**13a**) afforded APTs (i.e., **6**). In this case, the C2-NH_2 is more reactive than the C4-NH_2 due to a weaker inductive effect of the Me function. The chemical shifts assigned to the Me groups in APTs and AOTs, however, show no apparent difference.

3. Biological results and discussion

3.1. In vitro cytotoxicity

Our previous study has demonstrated that 5-(9-acridinylamino)toluidine derivatives bearing $CONHCH_2CH_2NMe_2$ and Me substituents at C4 and C5 of the acridine moiety, respectively, exhibited remarkable cytotoxicity both in vitro and in vivo, while, the simple 5-(9-acridinylamino)toluidines were less cytotoxic than the parent AHMA.[16] The results demonstrated that the electronegativity on the anilino ring, the substituent(s) on the acridine chromophore, as well as the hydrophobicity of the molecule, greatly influenced the cytotoxicity of 9-anilinoacridines. In the present studies, we evaluated the cytotoxic effects of target (9-acridinylamino)anisidines (AMAs, **14a**–**e**; AOAs, **15a**–**e**; and APAs, **17a**–**e**) against numerous human tumor cell (including mouth KB, nasopharyngeal carcinoma HONE-1, lung adenocarcinoma H460, colon HT-29, gastric carcinoma TSGH, hepatoma Hepa-G2, brain tumor DBTRG, and breast carcinoma MX-1) growth in culture (Table 1). The data clearly revealed that AOA **15e** was the most potent compound among the 5-(9-acridinylamino)anisidines bearing $CONCH_2CH_2NMe_2$ and Me substituents (AMA **14e**, AOA **15e**, and APA **17e**) at C4 and C5, respectively. The order of cytotoxicity was as follows:



Scheme 1. Reagents and reaction conditions: (i) 4-methylmorpholine (or catalytic concd HCl)/EtOh/$CHCl_3$, −10–0°C, 1–5; (ii) 5% Pd/C, H_2, Catalytic concd HCl, 50 psi, 25 min. (a) $R^1 = R^2 = H$; (b) $R^1 = Me$, $R^2 = H$; (c) $R^1 = CONHCH_2CH_2NMe_2$, $R^2 = H$; (d) $R^1 = CONHMe$, $R^2 = Me$; (e) $R^1 = CONHCH_2CH_2NMe_2$, $R^2 = Me$.

Table 1. The cytotoxicity of 5-(9-acridinylamino)anisidine derivatives on the inhibition of growth various human tumor cells in vitro

Compound	Inhibition of cell growth (IC_{50}, μM)							
	KB	HONE-1	H460	HT-29	TSGH	HEPA-G2	DBTRG	MCF-7
1 (AHMA)	ND[a]	0.30	ND[a]	0.90	0.50	1.40	3.50	ND[a]
3	ND[a]	0.04	ND[a]	0.12	0.40	0.20	0.16	ND[a]
AMT4	ND[a]	3.40	ND[a]	3.50	7.20	3.20	10.0	ND[a]
14a	3.20	3.32	2.57	3.00	2.96	3.56	4.70	4.89
14b	2.83	2.90	1.45	2.78	2.67	3.24	5.00	4.15
14c	0.70	0.73	0.44	0.47	0.38	0.41	1.69	1.16
14d	2.71	3.11	2.06	2.73	2.83	3.91	14.77	6.67
14e	0.09	0.06	0.03	0.05	0.03	0.04	0.18	0.18
AMT5	ND[a]	0.09	ND[a]	0.09	0.12	0.06	2.8	ND[a]
AOT8	ND[a]	0.90	ND[a]	2.50	2.50	4.0	4.30	ND[a]
15a	0.32	0.33	0.33	0.34	0.38	0.35	2.93	2.08
15b	0.32	0.34	0.29	0.38	0.38	0.36	2.45	2.60
15c	0.31	0.15	0.08	0.12	0.14	0.15	1.32	1.19
15d	0.39	0.50	0.19	0.60	0.62	0.97	2.33	3.24
15e	0.03	0.03	0.01	0.02	0.03	0.02	0.08	0.01
AOT9	ND[a]	0.09	ND[a]	0.09	0.08	0.10	0.30	ND[a]
APT6	ND[a]	0.80	ND[a]	2.50	4.10	2.50	4.00	ND[a]
17a	6.45	6.50	3.31	7.79	8.28	5.93	5.77	7.92
17b	6.36	4.82	2.40	8.62	7.73	10.75	21.67	26.19
17c	0.88	2.12	0.36	0.88	0.91	2.06	1.59	3.37
17d	2.55	3.07	0.43	4.53	3.32	8.00	14.88	17.50
17e	0.13	0.10	0.02	0.27	0.10	0.29	2.23	2.00
APT7	ND[a]	0.07	ND[a]	0.05	0.04	0.10	0.86	ND[a]

[a] Not determined.

AOA **15e** > AMA **14e** > APA **17e**. Among compounds with or without other substituent(s) on the acridine chromophore (i.e., AMA **14a–d**, AOAs **15a–d**, and APAs **17a–d**), the C4-$CONH_2NH_2NMe_2$-substituted derivatives (i.e., AMA **14c**, AOA **15c**, and APA **17c**) were approximately 2- to 10-fold more potent than their corresponding parent AMA **14a**, AOA **15a**, and APA **17a**, which depends on the tumor cell lines tested. The C4-Me-substituted (i.e., AMA **14b**, AOA **14b**, and APA **17b**) and C4-CONHMe, C5-Me-disubstituted compounds (i.e., AMA **14d**, AOA **15d**, and APA **17d**) did not have any improved cytotoxic effect in comparison with their corresponding parent compounds **14a**, **15a**, and **17a**, respectively. This demonstrated that the $CONH_2NH_2NMe_2$ substituent played an important role for their antitumor activity, perhaps due to better binding affinity with DNA double strands.[8,16] In general, the order of potency in the series of 5-(9-acridinylamino)anisidines was as follows: AOAs > AMAs > APAs. To elicit the effect of the OMe versus Me substituent attached on the anilino ring, we compared the cytotoxicity of 5-(9-acridinylamino)anisidines with those of previously synthesized AHMAs (**1** and **3**) and 5-(9-acridinylamino)toluidines (AMTs, **4**, **5**; AOTs, **6**, **7**; and APTs, **8**, **9**). The structure–activity relationship studies of these agents concluded that: (1) Both AMA **14e** and AOA **15e** were more cytotoxic than AHMA derivative **3**, while APA **17e** was less potent than **3** and (2) The degree of cytotoxicity was AMAs > AMTs; AOAs > AOTs; APTs > APAs. Our previous study had demonstrated that APTs were as potent as AOTs, but were significantly more cytotoxic than AMTs, with the exception of those compounds having Me and $CONH_2NH_2NMe_2$ substituents on the acridine chromophore (i.e., **5**, **7**, **9**, **14e**, **15e**, and **17e**). These findings have suggested that the Me group (possessing an inductive effect) and the electron-donating OMe group affect the electronegativity of the anilino ring and consequently alter the drug/enzyme-binding and antitumor activity. The effect of the substituent on the cytotoxicity of 9-anilinoacridines can be clearly observed in APT and APA families. The reason for the low cytotoxicity of APAs (especially, APA **17a–d**) remains unclear. Nevertheless, these studies have demonstrated that the drug/DNA binding of 9-anilinoacridines appears to be one of the most important factors for increasing the 9-anilinoacridine's cytotoxicity, as exhibited by **14e**, **15e**, and **17e**'s potent antitumor activity in vitro.

We evaluated further the effect of the newly synthesized compounds on cells overexpressing P-gp170/MDR1[18] and MRP (multidrug resistance-associated protein).[19] As shown in Table 2, the cytotoxicity of **14a**, **14e**, **15a**, **15e**, **17a**, and **17e** toward KB-Vin10 and KB-7D cells, which displayed overexpression of MDR1, MRP, and down-regulation of topoisomerase II, respectively, was similar to that of parental KB cells, indicating that 5-(9-acridinylamino)anisidines were not cross-resistant to etoposide-resistant (KB-7D) and vincristine-resistant (KB-Vin10) cells.

3.2. In vivo therapeutic activity

Among the agents tested, compound **15e** exhibited the most potent cytotoxic effect toward numerous human cancer cell lines and was subjected to further in vivo antitumor evaluation. The therapeutic efficacy of compound **15e** [maximal dose: 20 mg/kg (Q2D × 9); intravenous injection] on nude mice ($n = 4$) bearing human breast carcinoma MX-1 xenograft resulted in 60% reduction of tumor with 20% of body weight decrease compared with the control ($n = 5$) on day 26. Cain

Table 2. The cytotoxicity of 5-(9-acridinylamino)anisidine derivatives against human tumor KB and its sublines resistant to etoposide (KB-7D) and vincristine (KB-Vin10) cell growth in vitro

Compound	Inhibition of cell growth (IC_{50}, μM)			
	KB	KB-7D	KB-Vin10	PLDB (%) at 5 μM
AHMA	0.50	1.50 (3.0×)[a]	0.70 (1.4×)[a]	12.0
14a	2.45	3.06 (1.25×)	2.75 (1.12×)	0.0
14e	0.08	0.21 (2.65×)	0.25 (3.13×)	1.2
15a	0.31	0.25 (0.80×)	0.23 (0.74×)	6.5
15e	0.02	0.03 (1.50×)	0.04 (2.00×)	0.6
17a	6.0	7.1 (0.18×)	7.0 (1.16×)	4.6
17e	0.12	0.29 (2.42×)	0.32 (2.67×)	4.3
VP-16	0.40	78.8 (197×)	31.5 (78.8×)	ND[b]
Vincristine	0.0012	5.6 (4667×)	75.8 (65417×)	ND[b]

[a] Numbers in the brackets are folds of resistance of the resistant cells when compared with the IC_{50}'s of the KB parent cells.
[b] Not determined.

et al. indicated that increasing the electronic effect of substituents on the anilino ring of 9-anilinoacridines increased vulnerability for thiol attack,[20,21] thereby decreasing its cytotoxicity. This effect may be due to instability of the anisidine moiety in AOAs and APAs. These agents could be easily bio-oxidized to form relatively inactive compounds (i.e., *ortho*- or *para*-iminoquinone derivative, respectively).

3.3. Interaction of AHMA, APT, and AMT derivatives with topoisomerase II

In our previous study, AHMA and its analogs were shown to be potent Topo II inhibitors.[14,15] To study whether these compounds also inhibited Topo II catalytic activity, an ATP-dependent Topo II-mediated DNA relaxation assay was performed. The results for the representative compounds are shown in Figure 2, where AHMA, AMTs, and APTs bound tightly to the DNA at concentrations of 5–25 μM. Since Topo II inhibitors are capable of inducing double-stranded breaks, an in vitro K-SDS co-precipitation assay for measuring the amount of protein-linked DNA breaks (PLDBs) induced by AHMA, **14a**, **14e**, **15a**, **15e**, **17a**, and **17e**, was performed. After a 30 min exposure to an increased concentration of these compounds, except for compound **15b**, steady-state levels of PLDBs were increased in a dose-dependent manner, followed by a plateau at a concentration of 5 μM (data not shown). It reveals that there is a slight difference in PLDB level in the AMA compounds (0.0 and 1.2% for **14a** and **14e**, respectively) and no difference among APA compounds (4.6 and 4.3% for **17a** and **17e**, respectively). However, the amount of PLDBs generated by **15a** was significantly higher than **15e**. Thus, a direct correlation between the PLDB values of compounds tested and their cytotoxicity was not seen. A similar result was found in the series of (9-acridinylamino)toluidine derivatives.[16]

3.4. Interaction of AHMA, APT, and AMT derivatives with DNA

To study the correlation between cytotoxicity and the DNA-binding affinity of AHMA, APTs, and AMTs, a DNA circle-ligation assay with linearized DNA and T4 ligase was performed. The assay detected tertiary structure changes resulting from DNA binding to both

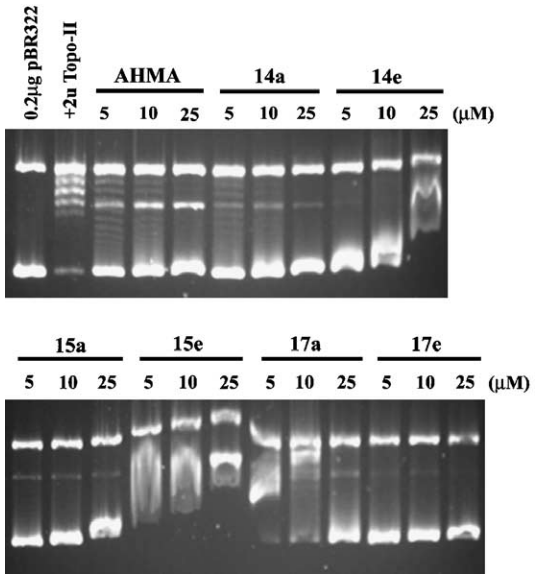


Figure 2. Inhibition of the DNA topoisomerase II catalytic activity by 5-(9-acridinylamino)anisidine derivatives. The experiment was performed by the method described previously.[26]

intercalating and non-intercalating compounds. The result showed that AHMA caused a dose-dependent band shift and produced a positive supercoiled DNA at concentrations greater than 2.5 μM (Fig. 3), indicating a change in the drug/DNA-linking number. Additionally, the DNA remained unchanged in the presence of **14e**, **15e**, or **17e** at 10 μM, suggesting that there was strong DNA intercalation and T4 ligase inhibition in the presence of these agents. The DNA-binding affinities for **14e**, **15e**, or **17e** are greater than those for **14a**, **15a**, and **17a**, respectively, and were more cytotoxic than the other tested compounds. The present results together with previous findings have demonstrated that increase in drug/DNA-binding activity enhanced the cytotoxicity of 9-anilinoacridines.

4. Conclusion

We have synthesized a series of 5-(9-acridinylamino)anisidine derivatives for antitumor studies against a

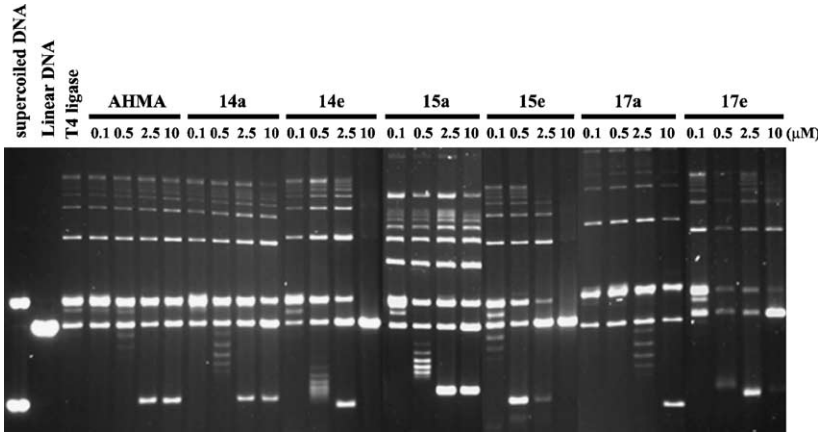


Figure 3. Effect of DNA unwinding by 5-(9-acridinylamino)anisidine derivatives measure, as described previously.[28,29]

variety of human tumor cell growth in vitro. AOT **15e** appeared to be the most potent agent among the compounds tested in this series. The SAR studies showed that compounds bearing $CONCH_2CH_2NMe_2$ and Me substituents (AMA **14e**, AOA **15e**, and APA **17e**) at C4 and C5 of the acridine chromophore, respectively, displayed potent cytotoxic effect in inhibiting growth of various human tumor cells in culture. Additionally, the present studies have demonstrated that the most important factor that affects the cytotoxicity of 5-(9-acridinylamino)anisidine (or 9-anilinoacridines) is the drug/DNA-binding affinity. However, electronegativity generated by the substituent (Me or OMe) on the anilino ring and hydrophobicity of the drug may have some effect on the drug's biological activity. The current systematic SAR studies will be beneficial for designing future 9-anilinoacridines with increased potent antitumor therapeutic efficacy.

5. Experimental

5.1. Materials and methods

Melting points were determined on a Fargo melting point apparatus and are uncorrected. Column chromatography was carried out on silica gel G60 (70–230 mesh, ASTM, Merck and 230–400 mesh, Silicycle Inc.). Thin-layer chromatography was performed on silica gel G60 F_{254} (Merck) with short-wavelength UV light for visualization. Elemental analyses were done on a Heraeus CHN-O Rapid instrument. ^{1}H NMR spectra were recorded in DMSO-d_6 solution on a 600 MHz Brucker AVANCE 600 DRX spectrometer, and chemical shifts are reported in ppm, relatively to TMS. Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

5.2. General procedure for the preparation of 5-(9-acridinylamino)anisidines

All new 5-(9-acridinylamino)anisidines (**14a**–**e**, **15a**–**e**, and **16a**–**e**) were prepared by the condensation of the requisite substituted 9-chloroacridines (**13a**–**e**) and the appropriate MeO-substituted 1,3-phenylenediamines (**10** and **11**) or 2-methoxy-5-nitroaniline (**12**) in a mixture of $CHCl_3$ and EtOH in the presence of 4-methylmorpholine (for **14a**–**e** and **15a**–**e**) or concd HCl (for **16a**–**e**) by following the method described previously.[14,16] The desired products were purified either by recrystallization or chromatography on a silica gel column. Compounds **17a**–**e** were synthesized by the reaction of **16a**–**e** under catalytic hydrogenation (5% Pd/C, H_2) in EtOH solution in the presence of concd HCl. The synthesis of representative compounds is given below. The analytic data and yield of other new derivatives are shown in Table 3.

5.2.1. *N*-Acridin-9-yl-5-methoxybenzene-1,3-diamine (AMA 14a). A suspension of 3,5-diaminoanisole dihydrochloride (2.11 g, 10 mmol) and 4-methylmorpholine (4.8 mL, 43.6 mmol) in ethanol (30 mL) was stirred in an ice-methanol bath for 10 min. A solution of 9-chloroacridine (**13a**, 3.44 g, 16 mmol) in $CHCl_3$ (50 mL) was then added dropwise to the above mixture and stirred at −5 °C for 2 h and then at room temperature overnight. The resulting solid product was collected by filtration and then recrystallized from EtOH to give **14a**, 2.24 g (71%); mp 235–236 °C; ^{1}H NMR δ 3.84 (3H, s, OMe); 5.58 (2H, br s, NH_2), 6.17 (1H; s, ArH), 6.23 (2H, br s, 2× ArH); 7.45 (2H, m, 2× ArH), 7.97 (2H, m, 2× ArH); 8.11 (2H, m, 2× ArH), 8.35 (2H, m, 2× ArH). Anal. ($C_{20}H_{17}N_3O{\cdot}HCl{\cdot}0.2H_2O$) C, H, N.

By following the same procedure as that for the synthesis of **14a**, compounds **14b**–**e** and **15a**–**e** were prepared.

5.2.2. *N*-Acridin-9-yl-4-methoxybenzene-1,3-diamine (APA 17a). A mixture of **16a** (1.15 g, 3.0 mmol) and 5% Pd/C in methanol (250 mL) containing concd HCl (0.5 mL) was hydrogenated at 50 psi for 25 min. The mixture was filtered through a pad of Celite and the solid cake was washed with methanol. The filtrate and washing were combined and evaporated under reduced pressure to dryness. The residue was chromatographed on a silica gel column (2 × 24 cm) using $CHCl_3$/MeOH (10:1 v/v) as the eluent. The product **17a** was eluted from $CHCl_3$/MeOH (5:1 v/v). Fractions containing **17a** were combined and concentrated under reduced pressure. The res-

Table 3. Analytic data and yield of 5-(9-acridinylamino)anisidines

Compound	Chemical formula*	mp (°C)	Yield (%)	Analysis
14a	$C_{20}H_{17}N_3O{\cdot}HCl{\cdot}0.2H_2O$	235–236	71	C, H, N
14b	$C_{21}H_{19}N_3O{\cdot}2HCl{\cdot}H_2O$	224–225	69	C, H, N
14c	$C_{25}H_{27}N_5O_2{\cdot}4HCl{\cdot}0.5H_2O$	257–258	55	C, H, N
14d	$C_{23}H_{22}N_4O_2{\cdot}0.25HCl{\cdot}0.25H_2O$	224–225	77	C, H, N
14e	$C_{26}H_{29}N_5O_3{\cdot}4HCl{\cdot}2.5H_2O$	207–208	45	C, H, N
15a	$C_{20}H_{17}N_3O{\cdot}2.5HCl{\cdot}0.8H_2O$	283–284	91.4	C, H, N
15b	$C_{21}H_{19}N_3O{\cdot}2HCl{\cdot}0.5H_2O$	280–283	86	C, H, N
15c	$C_{25}H_{27}N_5O_2{\cdot}5HCl{\cdot}3.2H_2O$	221–222	58.6	C, H, N
15d	$C_{23}H_{22}N_4O_2{\cdot}HCl{\cdot}3.7H_2O$	220–221	53	C, H, N
15e	$C_{26}H_{29}N_5O_2{\cdot}3HCl{\cdot}3.6H_2O$	173–174	75	C, H, N
16a	$C_{20}H_{15}N_3O{\cdot}HCl{\cdot}0.1H_2O$	239–240	90	C, H, N
16b	$C_{20}H_{15}N_3O_3{\cdot}0.8HCl{\cdot}1.8H_2O$	207–208	74	C, H, N
16c	$C_{25}H_{25}N_5O_4{\cdot}HCl{\cdot}2H_2O$	185–186	72	C, H, N
16d	$C_{23}H_{22}N_4O_2{\cdot}1.53HCl{\cdot}0.25H_2O$	254–255	74	C, H, N
16e	$C_{26}H_{27}N_5O_4{\cdot}2HCl{\cdot}2H_2O$	208–209	73	C, H, N
17a	$C_{20}H_{17}N_3O{\cdot}HCl{\cdot}0.6H_2O$	243–244	70	C, H, N
17b	$C_{21}H_{19}N_3O{\cdot}5HCl{\cdot}0.5H_2O$	230–231	84	C, H, N
17c	$C_{25}H_{27}N_5O_2{\cdot}2.5HCl{\cdot}4.1H_2O$	207–208	51	C, H, N
17d	$C_{23}H_{22}N_4O_2{\cdot}2.5HCl.0.8H_2O$	278–279	83	C, H, N
17e	$C_{26}H_{29}N_5O_2{\cdot}4HCl{\cdot}3.5H_2O$	229–230	72	C, H, N

* All compounds are hygroscopic and contain HCl and crystal water.

idue was treated with 4.2 N HCl/EtOAc (3 mL) and then evaporated in vacuo to dryness. The residue was co-evaporated several times with EtOH and the solid residue was recrystallized from EtOH/acetone to give **17a**, 667 mg (70%); mp 243–245 °C; ^{1}H NMR δ 3.36 (3H, s, OMe), 6.73 (1H, m, ArH), 6.78 (1H, m, ArH), 6.96 (1H, m, ArH), 7.39 (2H, m, ArH), 7.94 (2H, m, ArH), 8.05 (2H, m, 2× ArH), 8.27 (2H, m, 2× ArH), 10.36 (2H, br s, exchangeable, NH_2), 11.49 (1H, br s, exchangeable, NH). Anal. ($C_{20}H_{17}N_3O{\cdot}HCl{\cdot}0.6H_2O$) C, H, N.

By following the same procedure as that for the synthesis of **17a**, compounds **17b**–**e** were prepared.

5.3. Biological assays

5.3.1. Cytotoxicity assays. The effects of the compounds on cell growth were determined in all human tumor cells (i.e., colon HT-29, nasopharyngeal carcinoma HONE-1 and BM-1, hepatoma Hepa-G2, breast carcinoma MX-1, gastric carcinoma TSGH, brain tumor DBTRG, oral carcinoma KB, breast carcinoma MCF-7 and MX-1, and T-cell acute lymphocytic leukemia CCRF-CEM), for a 72 h incubation, by the XTT-tetrazolium assay, as described by Scudiero et al.[22] 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,[23,24] using a computer program on an IBM PC workstation.[25]

5.3.2. Inhibition of topoisomerase II catalytic activity by drugs. Topo-II catalytic activity was assayed by the ATP-dependent relaxation of pBR322 supercoiled DNA.[26] Various concentrations of the drugs were incubated with 2 U DNA topoisomerase II (Topogen) and 0.25 μg pBR322 DNA. The inhibition of relaxation activity was determined by comparison with an untreated control. AHMA was used as a positive control.

5.3.3. Measurement of protein-linked DNA breaks. Cells in log-phase growth were labeled with [^{14}C]thymidine for 24 h. After labeling, the cells were trypsinized, resuspended in fresh medium at a density of 5×10^5 cells/mL, and shaken gently in a 37 °C water bath for 1 h in suspension. Various concentrations of drugs were added and incubation was continued for an additional 0.5 h. The cells were collected and analyzed for protein-linked DNA breaks by potassium-sodium dodecyl sulfate (K-SDS) precipitation method, as described previously.[27] The percentage of PLDBs generated by the compounds was calculated by radioactivity (cpm) of precipitated DNA divided by the total radioactivity (cpm).

5.3.4. DNA unwinding measurement. The DNA unwinding effect of drugs was assayed according to the method described by Camilloni et al.[28] Briefly, 20 μg of pBR322 DNA was linearized with *Hin*dIII restriction endonuclease and recovered by phenol/chloroform extraction and ethanol precipitation. The reaction mixtures (totally 200 μL) containing 66 mM Tris–HCl (pH 7.6), 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.7 mM ATP, 0.6 μg DNA, and drugs were equilibrated at 15 °C for 10 min and then incubated with excess amount of T4 DNA ligase at 15 °C for 60 min. The reaction was stopped by the addition of 20 mM EDTA. DNA was analyzed by agarose-gel electrophoresis after treatment to remove the drugs from the reaction mixture: extraction with phenol and ether, and precipitation with ethanol. DNA was resuspended in 25 μL TE buffer with 1% SDS and analyzed in 1% agarose gel in TAE overnight.[29]

5.3.5. In vivo assay. Athymic nude mice bearing the nu/nu gene were used for human breast tumor MX-1 xenograft. Outbred Swiss-background mice were ob-

tained from Chares River Breeding Laboratories. Male mice 7 weeks old or older weighing 22 g or more were used for experiments. Drug was administered via tail vein by iv injection. Tumor volumes were assessed by measuring the length × width × height (or width) by using caliper. Vehicle used was 20 μL DMSO, diluted with 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 Institutional Animal Care and Use Committee.

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Supplementary data

The ^{1}H NMR spectroscopic data of compounds listed in Table 3 can be found, in the online version, at doi:10.1016/j.bmc.2005.07.018.

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