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New Analogues of AHMA as Potential Antitumor Agents: Synthesis and Biological Activity

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Abstract—A series of new analogues of 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **1**) and AHMA-ethylcarbamate (**2**) were synthesized by introducing an *O*-alkylcarboxylic acid esters to the CH_2OH function, displacing the CH_2OH function with a dimethylaminocarboxamido group or with a methyl function introduced at the *meta*-, *para*- or *ortho*-position to the NH_2 group to form 5-(9-acridinylamino)-*m*-toluidines (AMTs), 5-(9-acridinylamino)-*p*-toluidines (APTs) or 5-(9-acridinylamino)-*o*-toluidines (AOTs), respectively. The inhibitions of a variety of human tumor cell growth, interactions with DNA as well as inhibitory effect against topoisomerase II (Topo II) of these new agents were studied. Among AMT, APT and AOT derivatives with dimethylaminoethylcarboxamido and Me at C4 and C5 of acridine moiety (i.e., **21c**, **23c** and **26c**) were more cytotoxic than AHMA (**1**) and AHMA-ethylcarbamate (**2**), depending upon the tumor cell line tested. Detailed structure–activity relationships of the new analogues were studied.

Introduction

Previous studies on the development of potential 9-anilinoacridine congeners, 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **1**, Chart 1) derivatives, demonstrated that these compounds exhibited both in vitro and in vivo potent antitumor efficacy.[1,2] AHMA displayed better antitumor efficacy than *m*-amsacrine (*m*-AMSA, **6**) and VP-16 in mice bearing mammary carcinoma. For Lewis lung carcinoma, AHMA was as potent as VP-16, but more active than *m*-ASMA. We also reported that the alkylcarbamates of AHMA (AHMA-alkylcarbamates) were more effective than their corresponding parent AHMA derivatives. Of these compounds, AHMA-ethylcarbamate (**2**), 4′-methyl-AHMA-ethylcarbamate (**3**), and 4′-*N*,*N*-dimethyl-ethylcaboxamido-5′-methyl-AHMA-ethylcarbamate (**5**) possessed significant cytotoxicity against human leukemic HL-60 cell growth in culture.[2] Further in vivo studies revealed that AHMA-alkylcarbamates displayed significant anticancer therapeutic effects in mice bearing sarcoma 180, Lewis lung carcinoma and P388 leukemia.

The rational drug design for AHMA derivatives was based on the study of the metabolic pathway of *m*-AMSA (**6**), which was easily bio-oxidized to form *m*-AMSA-quinonediimine (*m*-AQDI) and had short half-life in human plasma.[3–5] The substituents (NH_2 and CH_2OH) on the anilino ring of AHMA, however, were located in the *meta*-position to each other, thus preventing oxidation. Therefore, AHMA compared to *m*-AMSA had a longer half-life in human plasma.[2] The previous structure–activity relationship (SAR) studies focused on the modification of the substituent(s) on the NH_2 and/or CH_2OH of the anilino ring. We demonstrated that the addition of the short acetyl group or the longer levulinyl function to the NH_2 and/or CH_2OH of the anilino ring slightly increased the cytotoxicity of the parent compound by inhibiting human leukemic HL-60

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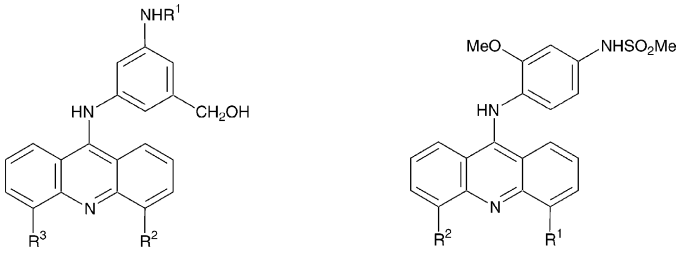


1 $R^1 = R^2 = R^3 = H$ (AHMA)
2 R^1 = COOEt, $R^2 = R^3 = H$
3 R^1 = COOEt, R^2 = Me, $R^3 = H$
4 $R^1 = H$, $R^2 = CONHCH_2CH_2NMe_2$, R^3 = Me
5 R^1 = COOEt, $R^2 = CONHCH_2CH_2NMe_2$, R^3 = Me

6 $R^1 = R^2 = H$ (*m*-AMSA)
7 R^1 = CONHMe, R^2 = Me (CI-921)

Chart 1. Potential 9-anilinoacridine congeners.

cell growth in culture.[1] Regarding the AHMA-alkylcarbamates, their cytotoxicity decreased with increasing length and size of the alkyl function.[2] The results indicated that both *N*-acyl-AHMA and AHMA-alkylcarbamates had better in vitro cytotoxicity than their corresponding parent AHMA derivatives. These findings, combined with the results from other laboratories,[6,7] demonstrated the importance of the lipophilicity/hydrophilicity balance in affecting the cytotoxicity of 9-anilinoacridines.

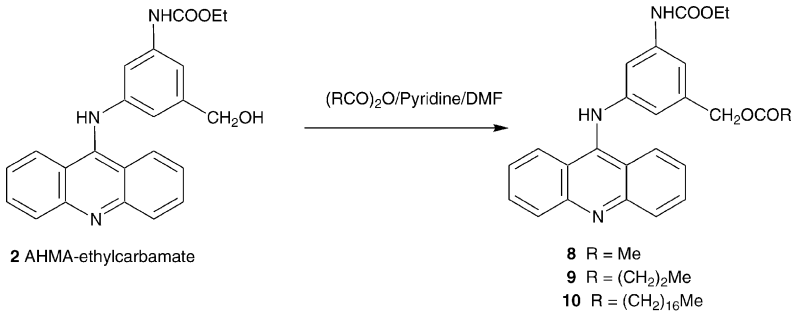
The drug–DNA binding affinity is another factor that may affect the antitumor activity of 9-anilinoacridines.[8–12] For example, the cytotoxicity of *m*-AMSA and AHMA were enhanced by introducing a alkylcarboxamido side chain and methyl group to the C4 and C5 position of the acridine ring, respectively, showing CI-921 (**7**) and **4** were more potent than *m*-AMSA (**6**) and AHMA (**1**), respectively.[2,10] In spite of these factors, there was no conclusive data to support the correlation between drug–DNA binding and its cytotoxicity. The cytotoxic effects of DNA topoisomerase II (Topo II) inhibitors were presumably due to trapping of DNA cleavable complex and result in drug–DNA–Topo II ternary complex[13–15] in which the acridine chromophore is considered as a DNA interactive domain and the aniline substituent is Topo II-binding domain. Therefore, aside from the drug–DNA binding enhanced by the substituent(s) on the acridine nucleus, one could visualize that the NH_2 and CH_2OH substituent(s) on the anilino ring of AHMA may engage in a critical interaction in the drug–Topo II or drug–DNA binding site to form drug–Topo II–DNA cleavable ternary complexes and that may demonstrate the superior antitumor activity of AHMA derivatives.

So far, we have studied the cytotoxic effects of the substituent(s) attached to the NH_2 and/or CH_2OH function(s) on the anilino ring or the substituent(s) on the acridine chromophore. The roles of these two functions need to be further explored. In this paper, we described the synthesis and detailed SAR of new 9-anilinoacridines. The study focused first on the modifications of the CH_2OH function of AHMA to increase its hydrophobicity. We synthesized *O*-acyl derivatives of AHMA-ethylcarbamate (alkylcarboxylic acid ester) to investigate whether the further increased lipophilicity of these agents could enhance the cytotoxicity of AHMA-ethylcarbamate (**2**). We also displaced the CH_2OH function of **2** with alkylcarboxamido derivatives; bearing a-CONHR function, they may also be involved in the drug–DNA interaction. Additionally, we replaced the CH_2OH function of AHMA with Me group introduced at the *meta*-, *para*- or *ortho*-position to the NH_2 group to form 5-(9-acridinylamino)-*m*-toluidines (AMTs), 5-(9-acridinylamino)-*p*-toluidines (APTs) or 5-(9-acridinylamino)-*o*-toluidines (AOTs), respectively. The new target compounds will enable us to better understand how the hydrophobicity affect the cytotoxicity of AHMA and the role of the NH_2 and CH_2OH functions in AHMA's antitumor activity. Furthermore, in vitro cytotoxicity on the inhibition of various human tumor cell growths, DNA binding, and inhibitory effects against Topo II of these newly synthesized 9-anilinoacridines were studied. These results revealed that AMT, APT and AOT derivatives with substituents on the acridine moiety had superior antitumor activity than either AHMA (**1**) or AHMA-ethylcarbamate (**2**).

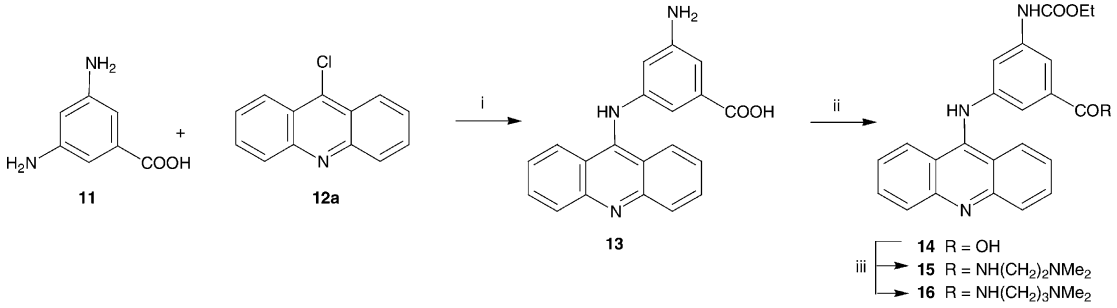
Results

Chemistry

The *O*-acyl derivatives of AHMA-ethylcarbamates, **8**, **9**, and **10**, were prepared in good to moderate yield by reaction of AHMA-ethylcarbamate (**2**) with acetic anhydride, *n*-butyric anhydride, and stearic anhydride, respectively, in pyridine (Scheme 1). The synthesis of 3-(9 - acridinylamino) - 5 - *N*,*N* - dimethylalkylcarboxamidoanilines (**15** and **16**) is shown in Scheme 2. By following the procedure previously described,[1,2] the condensation of 3,5-diaminobenzoic acid (**11**) with 9-chloroacridine (**12a**) in a mixture of $CHCl_3$/EtOH and *N*-methylmorpholine resulted in 3-(9-acridinylamino)-5-aminobenzoic acid (**13**) in 90% yield. Compound **13** was treated with ethyl chloroformate in pyridine to afford **14**, which was further reacted with *N*,*N*-dimethylethylenediamine or *N*,*N*-dimethyl-1,3-propanediamine in the



Scheme 1. Synthesis of the *O*-acyl derivatives of AHMA-ethylcarbamates.



Scheme 2. Reagents and conditions: (i) 4-methylmorpholine/$CHCl_3$/MeOH, 0 °C, 3 h; (ii) ClCOOR/pyridine/DMF, 0 °C, 2–3 h; (iii) $NH_2(CH_2)_2NMe_2$ or $NH_2(CH_2)_3NMe_2$/PyBOP/Hunig's base/DMF, 0 °C, 2 h.

presence of benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and Hünig's base in DMF to yield **15** and **16**, respectively.

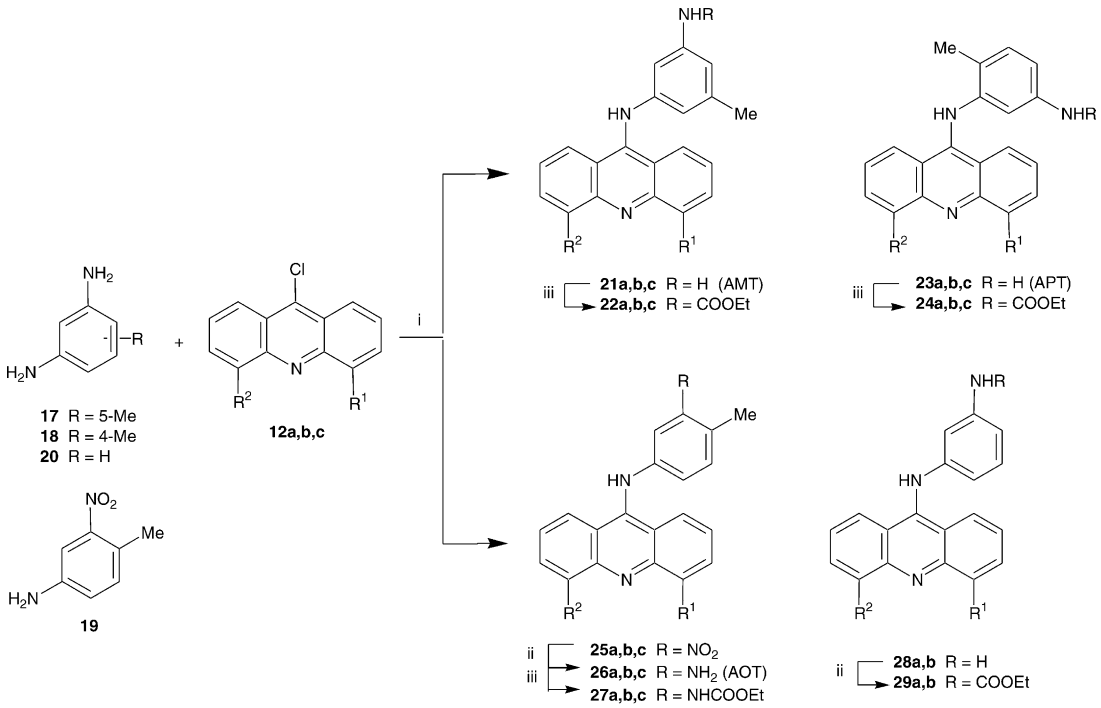
In a similar manner, the condensation of methyl substituted 1,3-phenylenediamines (**17**[16] or **18**) with the requisite 9-chloroacridines (**12a–c**) yielded 5-(9-acridinylamino)-*m*-toluidines (**21a–c**, AMTs) and 5-(9-acridinylamino)-*p*-toluidines (**23a–c**, APTs), respectively, in moderate to high yield (Scheme 3). It is noted that the reaction of **18** with **12a** may afford isomers **23a** and **26a**. The former (**23a**) was previously prepared by Denny et al.[8] by using the same starting materials under different conditions. To resolve the structure of **23a–c**, we synthesized their isomers, 5-(9-acridinylamino)-*o*-toluidines (**26a–c**, AOTs) by the condensation of 4-methyl-3-nitroaniline (**19**) with **12a–c**, followed by reduction of the nitro substituted intermediate **25a–c**. The target compounds **21a–c**, **23a–c**, and **26a–c** were finally treated with ethyl chloroformate in pyridine at 0 °C to afford the desired ethylcarbamate derivatives, AMT-ethylcarbamates (**22a–c**), APT-ethylcarbamates (**24a–c**), and AOT-ethylcarbamates (**27a–c**), respectively.

AHMA derivatives lacking the CH_2OH function, 3-(9-acridinylamino)aniline (**28a**)[17] and its 4′-Me substituted derivative (**29b**), were prepared by reaction of the known 1,3-diphenylamine (**20**) with 9-chloroacridines (**12a**,**b**) in good yield (80 and 72%, respectively), as shown in Scheme 3. By following the same approach, compounds **28a**,**b** were further converted into their ethylcarbamate derivatives (**29a**,**b**).

Biological Results and Discussion

In vitro cytotoxicity

Lipophilicity/hydrophilicity balance is a biologically important variable, where an appropriate adjustment in the lipophilic nature of drug may improve the drug's biological activity. A quantitative structure–activity relationships (QSARs) for antileukemic activity of simple carboxylic acid derivatives of 9-anilinoacridine was reported previously,[18] demonstrating that the addition of a Me group to the acridine moiety enhanced the antileukemic activity of **9**-anilinoacridine, due to the increased lipophilicity of the parent compound. Our previous study showed that the cytotoxicity of AHMA was enhanced by introducing an acyl group, such as acetyl or levulinyl function, to the NH_2 and/or CH_2OH groups.[1] We also demonstrated that all ethylcarbamate derivatives of AHMAs were more cytotoxic than their corresponding parent AHMA compounds in inhibiting cultured leukemic HL-60 cell growth, suggesting that adjustments of the lipophilic character of AHMA derivatives may improve the antitumor activity of compounds of this class. To optimize the cytotoxicity of AHMA derivatives, we synthesized carboxylic acid esters of AHMA-ethylcarbamates (**8**, **9**, and **10**) and evaluated their inhibitory effects against various human tumor cell growth in culture (Table 1). The results showed that the in vitro cytotoxic effect of AHMA-ethylcarbamate (**2**) on human nasopharyngeal carcinoma (HONE-1) and human brain tumor (DBTRG) was increased (4- to 10-fold) when an acetyl or *n*-butyryl function was introduced to the CH_2OH group (i.e., **8**



Scheme 3. Reagents and conditions: (i) 4-methylmorpholine (or concd HCl, catalytic amount)/$CHCl_3$/MeOH, 0 °C, 3 h; (ii) 5% Pd/C/H_2, concd HCl, 3 h; (iii) ClCOOEt/pyridine /DMF, 0 °C, 2–3 h, a series, $R^1 = R^2 = H$; b series, $R^1 = H$, $R^2 = Me$; c series, $R^1 = CONHCH_2CH_2NMe_2$, $R^2 = Me$.

Table 1. In vitro cytotoxicity of AHMA-ethylcarbamate derivatives against various human tumor cell growth

Compd	Inhibition of cell growth (IC_{50} μM)									Percentage of protein-linked DNA breaks[a]
	HT-29	HONE-1	TSGH	Hepa-G2	DBTRG	KB	MCF-7	MX-1	CCRF-CEM	
2	0.50	0.10	0.30	0.50	5.0	0.050	6.4	ND[b]	ND[b]	9.3
8	0.50	0.10	0.6	0.10	0.50	0.20	8.8	0.020	0.68	5.3
9	0.40	0.030	0.4	1.0	0.80	0.10	6.2	0.10	1.8	5.6
10	43	5.00	50	6.4	50	8.3	43	9.2	7.1	0.0
15	2.5	0.90	3.2	3.4	4.0	3.0	4.1	2.4	2.5	0.0
16	4.0	3.8	4.0	3.2	5.0	3.1	9.0	0.69	1.3	2.1

[a]The K-SDS coprecipitation assay: the percentage of protein-linked DNA breaks (PLDBs) generated by AHMA-ethylcarbamates at concentration of 5 μM.
[b]Not determined. Compound **1** is considered as a control (100%) (see Table 2).

and **9**, respectively). However, compounds **8** and **9** did not have an improved cytotoxic profile on the inhibition of other tumour cell growth (i.e., HT-29, TSGH, Hepa-G2, KB, MCF-7, CCRF-CEM, and MX-1) in culture. In contrast, the same studies revealed compound **10** having poor inhibitory effect on the tumor cell growth. This suggested that the cytotoxicity of **2** was increased with a small number of carbons on the carboxylic acid ester and was decreased by increasing the length of alkyl chain.

To study the role of the CH_2OH group on the AHMA-ethylcarbamates, we also prepared compounds **15** and **16** (Scheme 2), which bear an *N*,*N*-dimethylaminoalkyl-carboxamido chain instead of the CH_2OH group. One can envisage that the dissociable proton of the alkyl-carboxamido (-CONHR) function of compounds **15** and **16** may also involve in the drug–DNA interaction by means of formation a hydrogen bond. However, the results showed (Table 1) that both compounds were much less cytotoxic than **2**.

In another study, we replaced the CH_2OH group of AHMAs or AHMA-ethylcarbamates with a Me radical positioned at the *meta*- (i.e., AMT derivatives, **21a–c** and **22a–c**), *para*- (i.e., APT derivatives, **23a–c** and **24a–c**), or *ortho*-position (i.e., AOT derivatives, **26a–c** and **27a–c**) to the NH_2 group. The in vitro cytotoxicity of these derivatives was shown in Table 2. It demonstrated that APTs **23a–c** and AOTs **26a–c** were more cytotoxic than the corresponding AMTs **21a–c**. The 4′-CONH$(CH_2)_2NMe_2$-5′-Me substituted compounds, **21c**, **23c**, and **26c**, were approximately 2–18 times more potent than AHMA (**1**) or 2–10 times more cytotoxic than AHMA-ethylcarbamate (**2**) depending upon the type of tumor cell line tested. Compounds without substituent

Table 2. In vitro cytotoxicity of AMTs, APTs, AOTs and their ethylcarbamates against a variety of human tumor cell growth

Compd	Inhibition of cell growth (IC_{50} μM)					Percentage of protein-linked DNA breaks[a]
	HT-29	HONE-1	TSGH	Hepa-G2	DBTRG	
1	0.9	0.30	0.50	1.4	3.5	100
4	0.12	0.04	0.40	0.20	0.16	37.0
5	0.10	0.07	0.07	0.10	0.20	32.9
21a	3.5	3.4	7.2	3.2	10	12.3
21b	3.1	2.7	4.8	3.4	7.3	74.0
21c	0.09	0.09	0.12	0.06	2.8	0.0
22a	3.0	2.70	4.0	3.0	3.5	6.8
22b	2.6	2.2	3.6	2.9	4.6	2.7
22c	0.30	0.10	0.40	0.30	0.80	123.2
23a	2.5	0.80	4.1	2.5	4.0	116.4
23b	0.80	0.80	1.6	0.90	2.8	67.1
23c	0.05	0.07	0.04	0.10	0.86	0.0
24a	6.9	4.5	12	4.8	17	1.4
24b	3.7	3.9	8.3	4.4	9.2	13.6
24c	0.30	0.30	0.60	0.40	2.8	1.4
26a	2.5	0.9	2.5	4.0	4.3	5.5
26b	1.9	0.7	0.9	2.9	3.9	9.6
26c	0.09	0.09	0.08	0.1	0.3	4.1
27a	3.6	4.4	3.9	4.8	10	0.0
27b	3.8	3.9	4.2	4.4	8.2	0.0
27c	0.4	0.3	0.4	0.6	0.8	0.0
28a	3.8	3.1	4.5	4.0	8.5	57.5
28b	3.4	2.8	4.2	3.6	4.7	37.0
29a	3.6	3.8	7.8	4.1	9.3	42.5
29b	3.3	3.3	7.9	4.0	11	24.6

[a]The K-SDS coprecipitation assay: the percentage of protein-linked DNA breaks (PLDBs) generated by new 9-anilinoacridine derivatives at concentration of 5 μM. Compound **1** is considered as a control (100%).

or with a methyl function on the acridine ring (i.e., AMT **21a**,**b**, APT **23a**,**b**, and AOT **26a**,**b**) were generally less cytotoxic than **1** and **2**. In comparison with compounds bearing disubstituents on the acridine moiety, compounds **4**, AMT **21c**, APT **23c** AOT **26c** have comparable in vitro cytotoxicities. Of these compounds, **23c** was the most potent. Our previous study demonstrated that all ethylcarbamate derivatives of AHMA were more cytotoxic than the corresponding AHMAs in inhibiting human leukemic HL-60 cell growth in culture.[2] For further antitumor study, we reinvestigated the in vitro cytotoxicity of AHMAs and AHMA-ethylcarbamates (**1** vs **2** and **4** vs **5**), and compared them with AMTs (**21a–c** vs **22a–c**), APTs (**23a–c** vs **24a–c**), and AOTs (**26a–c** vs **27a–c**). Like AHMAs (**1** vs **2** and **4** vs **5**), AMT-ethylcarbamates (**22a–c**) were as potent as or slightly more cytotoxic than AMTs (**21a–c**) depending on the tumor cells tested. APT-ethylcarbamates (**24a–c**) and AOT-ethylcarbamates (**27a–c**), however, were much less cytotoxic than the corresponding parent compounds **23a–c** and **26a–c**, respectively, probably due to the steric effect by interfering drug-enzyme binding. On the other hand, the Me group in APTs and AOTs was located at the *para*- and *ortho*-position to the two amino functions in the anilino ring. The electron-negativity of the two amino groups in **23a–c** and **26a–c** is increased by the inductive effect of the Me group. Since the Me function in AMTs is located at the *meta*-position to the two amino functions, the electron-negativity of the two amino functions in AMTs (**21a–c**) is weaker than that of APTs and AOTs; suggesting that increasing the electron-negativity of the amino function on the anilino ring may enhance the drug–enzyme interaction and raise the 9-anilinoacridine's cytotoxicity. The introduction of the two substituents (i.e., Me and $CONHCH_2CH_2NMe_2$) to the acridine chromophore was thought to enhance the drug–DNA interaction.[2,10] It is also of great interest to note that the order of increasing cytotoxicity after introducing the two substituents was 1.2- to 22-fold for AHMAs (**1** vs **4**), 4- to 60-fold for AMTs (**21a** vs **21c**), 5- to 100-fold for APTs (**23a** vs **23c**), and 10 to 40-fold for AOTs (**26a** vs **26c**) thus confirming the benefit of methyl and *N*-(2-dimethylaminoethyl)carbamido substituents at the 5- and 4-positions of acridine chromophore, respectively. The results clearly demonstrated that the change in the cytotoxic profile by functional modifications in the anilino and/or acridine ring(s) might be attributed to the appropriate increasing lipophilicity, steric effect, and electron-negativity of the anilino ring for drug–DNA and drug–Topo II interaction.

Our previous report revealed that AHMA lacking the NH_2 function was 100-times less active than AHMA on the inhibition of human leukemia HL-60 cell growth in culture.[1] In the present study, we found that AHMA without the CH_2OH (compound **28a**,**b** and **29a**,**b**) were 3–10 times less cytotoxic than AHMA on the inhibition of the growth of various human tumor cell lines tested (Table 2). Although we did not directly compare the potency of these compounds with that of compounds lacking NH_2 function in the same tested system, the low cytotoxic **28a**,**b** and **29a**,**b** suggested that the CH_2OH group plays an important role in inhibitory activity of AHMA against the tumor cells and its binding to DNA.

To study whether AMT **21c**, APT **23c**, and AOT **26c** were cross-resistant with vinblastine or taxol, the growth inhibition of human lymphoblastic leukemic cells (CCRF-CEM) and its drug-resistant sublines (CCRF-CEM/VBL and CCRF-CEM/taxol, respectively) was evaluated (Table 3). The results demonstrated that **21c** and **23c**, and **26c** (either their trihydrochloride salt or free compounds) were far more potent than AHMA (**1**) and not cross-resistant with vinblastine or taxol, suggesting they were neither good *p*-glycoprotein substrates nor mutated tubulins.

Interaction of AHMA, AMT, APT and AOT derivatives with Topo II

AHMA was previously demonstrated to be a potent Topo II inhibitor.[1] The pattern of the Topo II-mediated cleavage of DNA induced by AHMA was similar to that of *m*-AMSA.[21] To study whether these compounds can stimulate Topo II-induced DNA cleavage, a Topo II-mediated DNA cleavage assay using ^{32}P-labeled pBR322 DNA as a substrate was performed and *m*-AMSA and AHMA were included as a positive control. As shown in Figure 1, the Topo II-mediated DNA breaks induced by *m*-AMSA and AHMA were in a dose-dependent manner, and could be more clearly observed at the concentration above 10 μM, whereas compounds **21c**, **23c** and **26c** induced Topo II-mediated DNA cleavages were only at concentration of 2.5 μM. No DNA cleavage was detected above the concentration of 5 μM indicating that these agents bound to DNA doubled strands tightly. We further study the inhibitory activity of newly synthesized AMT, APT and AOT derivatives against Topo II using ATP-dependent Topo II-mediated relaxation assay. The results of the representative compounds were shown in Figure 2. It revealed that AHMAs, AMTs, APTs and AOTs bind tightly to the DNA double helix at the concentration of 5–25 μM, resulting in difficult comparison between the drug's relative potency on Topo II inhibition. Since Topo II inhibitors induced double strand DNA breaks, the in vitro k-SDS co-precipitation assay was performed to measure the amount of protein-linked DNA breaks (PLDBs) caused by the newly synthesized 9-anilinoacridines and compared with AHMA (**1**). After a 30 min exposure to increasing concentrations of these compounds, steady-state levels of PLDBs were increased in a dose-dependent manner, followed by a plateau at concentration of 5 μM (data not shown). Nevertheless, the amount of PLDBs generated by compounds **4**, **5** and **16** decreased as the concentration was increased to 10 μM, suggesting that high concentrations of these agents changed the conformation of DNA and making difficult for Topo II to have access to the DNA. The amount of PLDBs generated by AHMA-ethylcarbamate derivatives (**2**, **8**–**10**, and **15**–**16**) at concentration of 5 μM was shown in Table 1, revealing the percentage of PLDBs decreased by AHMA-ethylcarbamates with increasing the length of the ester chain (**2** > **8**, **9** > **10**). However, in the carboxamido derivatives, the addition of one carbon unit, **16**, demonstrated a significant increase in PLDBs compared to **15**. Interestingly, in examining the AMTs, APTs, and AOTs, in general, there were no significant correlations between the structures of these compounds to PLDBs. For example, Table 2 showed that only AMT (**21b**, **22c**) and APT **23a**,**b** induced more PLDBs, while AMT **21c**, APTs (**24a**–**c**), AOT (**26a**–**c** and **27a**–**c**) had significantly less PLDBs and were poor Topo II inhibitors. As mentioned previously, disubstituted 9-anilinoacridines **4**, **5**, **21c**, **23c**, and **26c** were the most cytotoxic among compounds tested. Consequently, there was no correlation observed between the in vitro cytotoxicity of AMTs, APTs and AOTs and their

Table 3. Growth inhibition of CCRF-CEM human lymphoblastic leukemic cells and its drug-resistant sublines by AMT **21c**, APT **23c** and AOT **26c**

Compd	Inhibition of cell growth (IC_{50} μM)		
	CCRF-CEM	CCRF-CEM/VBL[a]	CCRF-CEM/taxol[b]
21c	0.043	0.094 [2.2 x][c]	0.044 [1.02 x]
23c	0.036	0.061 [1.7 x]	0.037 [1.03 x]
26c	0.079	0.057 [0.72x]	
AHMA (**1**)	1.133	0.673 [0.59 x]	1.12 [0.96 x]

[a]CCRF-CEM subcell line 329-fold resistant to vinblastine.
[b]ACCRF-CEM subcell line 40-fold resistant to taxol.
[c]Number in the brackets are folds of resistance when compare with the parent CCRF-CEM cell line.

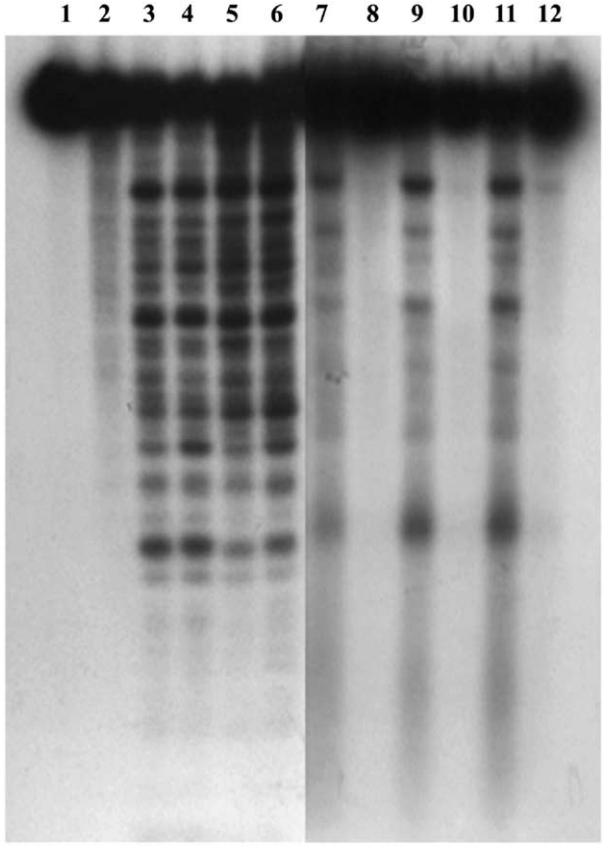


Figure 1. Effects of compounds on Topo II-dependent DNA double-stranded breaks. This assay was done as described,[27] stopped by SDS-proteinase K treatment, and then analyzed by neutral gel electrophoresis. Lane 1, control DNA; lane 2, DNA plus 15 units of Topo II; lanes 3 and 4, enzyme plus 10 and 50 μM *m*-AMSA, respectively; lanes 5 and 6, enzyme plus 10 and 50 μM AHMA, respectively; lanes 7 and 8, enzyme plus 2.5 and 5 μM compound **21c**, respectively; lanes 9 and 10, enzyme plus 2.5 and 5 μM compound **23c**, respectively; lanes 11 and 12, enzyme plus 2.5 and 5 μM compound **26c**, respectively.

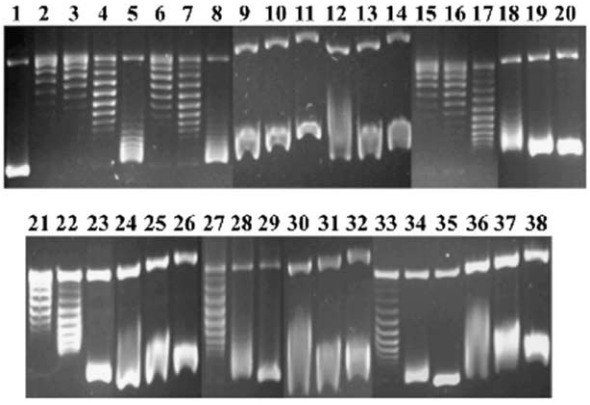


Figure 2. Inhibition of the DNA topoisomerase II catalytic activity by AHMA, AMT, APT and AOT derivatives. The experiment was performed by the method described previously.[26] Different concentrations (5, 10 and 25 μM) of the following compounds were tested: lane 1, DNA alone; lane 2, DNA plus 2 units of enzyme; lanes 3–5, compound **1**; lanes 6–8, compound **2**; lanes 9–11, compound **4**; lanes 12–14, compound **5**; lanes 15–17, compound **8**; lanes 18–20, compound **15**; lanes 21–23, compound **21a**; lanes 24–26, compound **21c**; lanes 27–29, compound **23a**; lanes 30–32, compound **23c**; lanes 33–35, compound **26a**; lanes 36–38, compound **26c**.

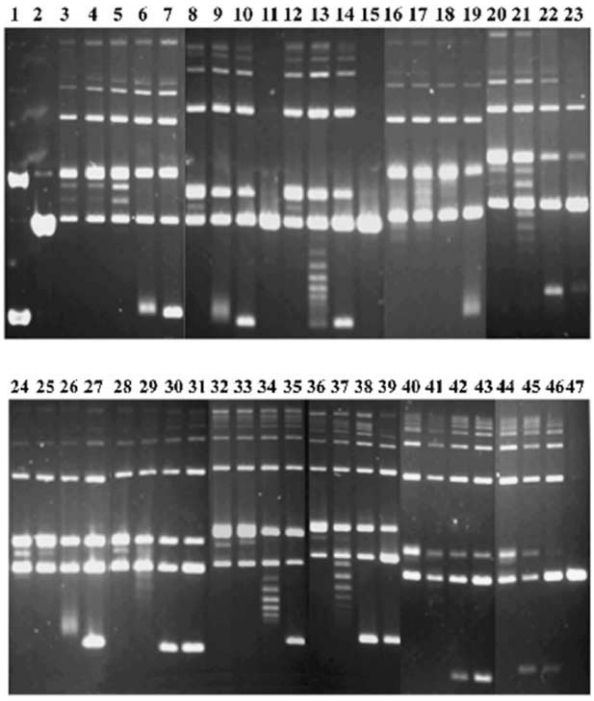


Figure 3. Effect of DNA unwinding by AHMA, AMT, APT and AOT derivatives measured as described previously.[14,28] Different concentrations (0.1, 0.5, 2.5 and 10 μM) of the following compounds were tested. Lane 1, supercoiled DNA; lane 2, linearized DNA; lane 3, linearized DNA plus T4 ligase; lanes 4–7, compound **1**; lanes 8–11, compound **4**; 12–15, compound **5**; lanes 16–19, compound **8**; lanes 20–23, compound **15**; lanes 24–27, compound **21a**; lanes 28–31, compound **21c**; lanes 32–35, compound **23a**; lanes 36–39; compound **23c**; lanes 40–43, compound **26a**; lanes 44–47, compound **26c**.

PLDBs values. It should be noted that lipophilicity of these agents may also affect the uptake of the drugs by the cells and hence their cytotoxicity.

Interaction of AHMA, AMT, APT and AOT derivatives with DNA

To investigate whether the DNA binding activity of AHMA, APT, and AMT derivatives correlated with their antitumor effect, a DNA circle-ligation assay with linearized DNA and T4 ligase was performed. The assay detected the tertiary structure alterations resulting from DNA binding of both intercalating and non-intercalating drugs. The results of the representative compounds were shown in Fig. 3. It revealed that AHMA (**1**, lanes 4–7) caused a concentration-dependent band shift and also produced a positive supercoiled DNA at concentration greater than 2.5 μM, indicating a change in the drug–DNA linking number. However, DNA remained unchanged in the presence of compound **4** or **5** at a concentration 10 μM, suggesting strong DNA intercalation and T4 DNA ligase inhibition occurred in the presence of these agents. All compounds tested, except **9** and **10**, caused DNA band shift with similar DNA binding affinity to that of AHMA, indicating that compounds **9** and **10** were weak DNA intercalators. As mentioned above, alkyl esters of AHMA-ethylcarbamate (**8** and **9**), AMT **22c**, APT **23c**, and AOT **26c** were more cytotoxic than other tested 9-anilinoacridines. Taken together, the current study also showed that there is no direct correlation between drug–DNA binding activity and cytotoxicity of 9-anilinoacridines.

Table 4. Therapeutic effect of AMT **21c**, APT **23c** and AOT **26c** in nude mice bearing human MX-1 mammary carcinoma xenograft[a,b]

Compd (mg/kg)	Average weight change (gm)								Average tumor volume (T/C)					
	n	Day 11	14	17	20	23	26	29	Day 11	14	17	20	23	26
Control	4	26.6	+0.9	+2.0	+1.7	+1.4	+0.2		1.00	1.00	1.00	1.00	1.00	1.00
21c														
10	4	25.8	+0.7	+1.1	+1.0	+1.8	+1.1	+1.1	0.81	0.54	0.32	0.24*	0.23*	0.27*
20		27.6	+1.2	+1.5	+1.2	+0.5	+0.2	−1.4	0.93	0.58	0.32	0.22*	0.22*	0.22*
23c														
5	4	27.6	+1.0	+1.6	+0.8	+1.1	+0.9	+0.4	1.01	0.71	0.50	0.44*	0.34*	0.44*
10	4	27.6	+1.1	+1.6	+0.8	+0.5	−0.8	−2.3	0.73	0.65	0.46	0.24*	0.21*	0.21*
26c														
20	3	32.8	+0.1	+0.3	+0.4	−0.5	−0.5	−1.1	0.44	0.38	0.45	0.43	0.33	0.31

[a]MX-1 tumor tissue 50 mg/mouse was implanted on day 0. Treatment started on day 11. Q3D×6 iv injection on day 11, 14, 17, 20, and 26.
[b]Tumor size of control group on day 11, 14, 17, 20, 23, and 26 were 138, 427, 1174, 2865, 5092, and 6125 mm^3, respectively. *$p < 0.05$ compared with control. Control mice were sacrificed on Day 26 due to excessive tumor burden.

In vivo therapeutic activity

The in vivo antitumor efficacy of AMT **21c**, APT **23c** and AOT **26c** using human breast tumor (MX-1) xenograft was also studied, as these three compounds exhibited the most cytotoxic toward different human cancer cell lines among agents tested. As shown in Table 4, mice were treated with **21c**, **23c** and **26c**, Q3D×6, via intravenous injection. Significant delay of tumor growth ($p < 0.05$) in the treated groups was observed. Approximately 70–80% of average tumor volume was reduced on day 26 when mice were treated with **21c**, **23c** and **26c** at the dose of 20, 10 and 20 mg/kg, respectively. No significant body weight changes between the control and treated mice were observed. Our unpublished result showed that compound **5** was toxic to the host (four mice died on day 21, 21, 21, and 22) at dose of 20 mg/kg in the same experiment. However, no drug-related deaths of the treated mice were noted when mice were treated **21c**, **23c** and **26c**, indicating low toxicity of these compounds to the host.

Conclusion

9-Anilinoacridines are a major class of DNA intercalating agents and Topo II inhibitors. These agents are capable of forming drug–DNA–Topo II ternary complexes.[13,14,20,21] The acridine chromophore of these compounds is proposed to be the DNA-binding domain, while the anilino moiety is the enzyme-binding domain in these ternary complexes.[2,18] Recently, quantitative structure–activity relationship (QSAR) studies for 9-anilinoacridines have been reviewed by Hansch et al.[22] Three major and consistent conclusions, including (1) the hydrophobicity of agent, (2) the electronic effects of substituent(s), and (3) the number of negative steric hindrances, were drawn from these quite diverse QSAR of the 9-anilinoacridines.

In the present studies, we have synthesized *O*-alkylcarboxylic acid esters of AHMA-ethylcarbamates (**8**, **9**, and **10**). It revealed that more lipophilic compounds **8** and **9** were more cytotoxic than AHMA-ethylcarbamate (**2**). However, the cytoxicity of **2** was markedly decreased when a longer carbon chain in the alkylcarboxylic acid ester were introduced. Both **8** and **9** were good inhibitors of Topo II, but not **10**; suggesting that longer ester chain may interfere with the drug–DNA binding and thus resulting in low cytotoxicity. We have also synthesized AMTs, APTs and AOTs and their ethylcarbamates to increase the lipophilicity of AHMA. The results showed that AMTs (**21a**,**b**), APTs (**23a**,**b**), and APTs (**26a**,**b**) were less cytotoxic than AHMA (**1**) and were poor Topo II inhibitors. Moreover, the AMT-ethylcarbamate derivatives, **22a**,**b**, structurally like AHMA-ethylcarbamate (**2**), were revealed to be slightly more cytotoxic than the corresponding parents **21a**,**b**, with the exception of compound **22c**, which was less cytotoxic than **21c**. In contrast, the APT-ethylcarbamates (**24a–c**) and AOT-ethylcarbamates (**27a–c**) were less active than their corresponding parents **23a–c** and **26a–c**, respectively. It demonstrated that simply making compounds more hydrophobic does not increase drug's cytotoxicity. However, addition of Me and CONHCH$_2$CH$_2$NMe$_2$ functions to the acridine chromophore of all newly synthesized derivatives dramatically increased the cytotoxicity of 9-anilinoacridine derivatives probably due to enhance the interaction between drug and DNA. The results clearly demonstrated that the lipophilicity/hydrophilicity balance and drug-DNA binding affinity alter the cytotoxicity of AHMA analogues.

We also demonstrated that APTs and AOTs were more cytotoxic than AMTs probably due to the inductive effect of the Me function. It suggested that APTs and AOTs might be more favorable to interact with Topo II. However, APT-ethylcarbamates (**24a–c**) and AOT-ethylcarbamates (**27a–c**) were found to be less active than their corresponding parents **23a–c** and **26a–c**, respectively. These findings imply that the ethylcarbamoyl side chain of APTs and AOTs may disrupt the drug-enzyme interaction. These results indicated that the electron-negativity and the steric effect of the substituent(s) on the anilino ring also affect the cytotoxicity of 9-anilinoacridines.

Our recent report illustrated that the in vitro cytotoxicity of AHMA derivatives linked to DNA minor groove binding agent (AHMA-*N*-netropsin and AHMA-*O*-netropsin derivatives) was increased with escalating PLDBs values.[19] However, this in not the case for AMTs, APTs, and AOTs. Some of 9-anilinoacridine derivatives still could increase Topo II-mediated DNA cleavage at low concentration in spite of low PLDBs values. Moreover, the AHMA derivatives presented in this paper displayed strong DNA binding. The inhibition of DNA relaxation in many cases may due to the effect of the compound on the structure of DNA as opposed to a specific effect on enzyme activity. Furthermore, we showed that no direct correlation between drug–DNA binding activity and cytotoxicity of 9-anilinoacridines, suggesting that the cytotoxicity of 9-anilinoacridines may be attributed to the effects of the inhibition of enzyme and intercalation of DNA.

In summary, the present work targets the effects of lipophilicity/hydrophilicity balance, steric effects on the drug–enzyme and drug–DNA binding by modifying the substituents on the anilino ring and/or acridine chromophore of AHMA derivatives. These factors need to be taken into account in order to optimize the cytotoxicity of 9-anilinoacridines. Among the newly synthesized 9-anilinoacridines described in this paper, AMT **21c**, APT **23c**, and **26c** exhibited in vitro cytotoxicity in inhibiting human tumor cell growth tested and displayed appreciably in vivo antitumor efficacy in nude mice bearing human MX-1 xenograft with less toxicity to the host. Moreover, these compounds were considerably more potent than AHMA **1** in inhibiting human lymphoblastic leukemic CCRF-CEM cell growth in culture and are not resistant to vinblastine- and taxol-resistant cells.

Experimental

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). 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 on a Brucker AMX 400 spectrometer with Me_4Si as the internal standard. The followings are the synthesis of the representative compounds. The analytic data and yield of other new derivatives are shown in Table 5.

***O*-Acetyl-AHMA-ethylcarbamate (8)**. A mixture of **2**[2] (420 mg, 1.08 mmol), acetic anhydride (0.4 mL, 4.2 mmol) and pyridine (0.4 mL, 5 mmol) in dry DMF (3.0 mL) was stirred at 0 °C for 3 h and then at room temperature for 42 h. The reaction mixture was then evaporated in vacuo to dryness and the solid residue was recrystallized from EtOH to give **8**: 300 mg (64.6%); mp: 215–217 °C; ^{1}H NMR (DMSO-d_6) δ 1.24 (3H, s, J = 6.7 Hz, Me), 2.02 (3H, s, Me), 4.12 (2H, t, J = 6.7 Hz, CH_2), 5.01 (2H, s, CH_2), 7.48 (4H, m, ArH), 8.12 (7H, m, ArH), 9.98 and 11.52 (each 1H, brs, NH). Anal. ($C_{25}H_{23}N_3O_4 \cdot 3.25H_2O$) C, H, N.

Derivatives **9** and **10** were prepared in a similar manner from **2** and the proper carboxylic acid anhydride.

General procedure for the preparation of 9-anilinoacridines

All new 9-anilinoacridine derivatives (**13**, **21a–c**, **23a–c**, and **28a**,**b**) were prepared by the condensation of the requisite substituted 9-chloroacridines (**12a–c**) and the appropriate 1,3-phenylenediamines (**11**, **17**, and **18**) in a mixture of $CHCl_3$ and EtOH in the presence of 4-methylmorpholine by following the method described previously.[1,2] The desired products were purified by either recrystallization or chromatography on a silica gel column. Compounds **25a–c** were synthesized by the reaction of **12a–c** and 3-nitro-*p*-toluiene (**19**) under acidic conditions using concd HCl as the catalyst instead of 4-methylmorpholine. The representative examples were described as follows.

3-(9-Acridinylamino)-5-aminobenzoic acid (13). To a mixture of 3,5-diaminobenzoic acid (**11**, 3.04 g; 20 mmol) and 4-methylmorpholine (4.4 mL, 40 mol) in EtOH (50 mL) a solution of 9-chloroacridine (**12a**; 4.27 g; 20 mmol) in $CHCl_3$ (20 mL) was added dropwise at an ice bath temperature. After being stirred for 1 h, the temperature was raised to room temperature and the mixture was stirred further 3 h. The precipitated orange product was collected by filtration, washed with EtOH and dried to give **13**, 6.84 g (94%). A sample was recrystallized from DMF/MeOH for elemental analysis: mp >280 °C; ^{1}H NMR (DMSO-d_6) δ 5.74 (2H, brs, NH_2), 6.78 (1H, s, ArH), 7.05 (1H, s, ArH), 7.23 (1H, s, ArH), 7.49 (2H, m, 2×ArH), 7.98–8.06 (4H, m, 4×ArH), 8.29 (2H, m, 2×ArH), 11.38 (1H, brs, NH), 12.93 (1H, brs, 1H, COOH). Anal. ($C_{20}H_{15}N_3O_2 \cdot 1.5H_2O$) C. H. N.

4-(9-Acridinylamino)-2-nitrotoluene hydrochloride (25a). A solution of **12a** (4.27 g, 20 mmol) in $CHCl_3$ (10 mL) was added dropwise to a mixture of 4-amino-2-nitrotoluene (**19**, 3.35 g, 22 mmol) and concd HCl (0.2 mL) in EtOH (20 mL) cooled in an ice bath. After the reaction mixture was stirred for 3 h, the solid was collected by filtration and was recrystallized from EtOH to give **25a**, 6.61 g (90%), mp >280 °C; ^{1}H NMR (DMSO-d_6) δ

Table 5. Analytic data and yield of the new compounds

Compound	Chemical formula[a]	mp (°C)	Yield (%)	Analyses
9	$C_{27}H_{27}N_3O_4 \cdot 3H_2O$	205–208	48	C, H, N
16	$C_{28}H_{31}N_5O_3 \cdot 2HCl \cdot 3H_2O$	217–219	64	C, H, N
21a	$C_{20}H_{17}N_3 \cdot 2HCl \cdot 1.75H_2O$	>280	85	C, H, N
21b	$C_{21}H_{19}N_3 \cdot HCl \cdot 0.13H_2O$	>280	61	C, H, N
21c	$C_{26}H_{29}N_5O \cdot 3HCl \cdot 3H_2O$	239–240	46	C, H, N
22b	$C_{24}H_{23}N_3O_2 \cdot HCl \cdot 1.3H_2O$	241–242	77	C, H, N
22c	$C_{29}H_{33}N_5O_3 \cdot 2HCl \cdot 1.3H_2O$	263–264	85	C, H, N
23a	$C_{20}H_{17}N_3 \cdot 2HCl \cdot 2H_2O$	>280	86	C, H, N
23b	$C_{21}H_{19}N_3 \cdot HCl \cdot 0.25H_2O$	>280	88	C, H, N
23c	$C_{26}H_{29}N_5O \cdot 3HCl \cdot 3H_2O$	228–229	57	C, H, N
24a	$C_{23}H_{21}N_3O_2 \cdot HCl \cdot 0.3H_2O$	258–259	64	C, H, N
24b	$C_{24}H_{23}N_3O_2 \cdot HCl \cdot 1.5H_2O$	248–149	71	C, H, N
24c	$C_{29}H_{33}N_5O_3 \cdot 3HCl \cdot 3H_2O$	182–183	80	C, H, N
25b	$C_{21}H_{17}N_3O_2 \cdot 3HCl \cdot 0.25H_2O$	>280	86	C, H, N
25c	$C_{26}H_{27}N_5O_3 \cdot 2HCl \cdot 3H_2O$	241–242	59	C, H, N
26b	$C_{21}H_{19}N_3 \cdot 2HCl \cdot 0.4H_2O$	>280	58	C, H, N
26c	$C_{26}H_{29}N_5O \cdot 4HCl \cdot 3.8H_2O$	245–246	86	C, H, N
27a	$C_{23}H_{21}N_3O_2 \cdot HCl \cdot 2H_2O$	201–202	52	C, H, N
27b	$C_{24}H_{23}N_3O_2 \cdot HCl \cdot H_2O$	242–243	53	C, H, N
27c	$C_{29}H_{33}N_5O_3 \cdot 3HCl \cdot 8.25H_2O$	131–132	80	C, H, N
28a	$C_{19}H_{15}N_3 \cdot 2HCl \cdot 1.75H_2O$	>280	80	C, H, N
28b	$C_{20}H_{17}N_3 \cdot 2HCl \cdot 1.25H_2O$	183–184	72	C, H, N
29a	$C_{22}H_{19}N_3O_2 \cdot HCl \cdot 0.3H_2O$	284–285	55	C, H, N
29b	$C_{23}H_{21}N_3O_2 \cdot 1.5HCl \cdot 1.25H_2O$	259–260	45	C, H, N

[a]Compounds are hydroscopic and contain crystal water.

2.57 (3H, s, Me), 7.50–7.61 (4H, m, ArH), 8.02–8.05 (2H, m, ArH), 8.00 (1H, m, ArH), 8.16–8.18 (1H, m, ArH), 8.30–8.34 (1H, m, ArH), 11.70 (1H, brs, NH). Anal. ($C_{20}H_{15}N_3O_2$·HCl·1.25H_2O) C, H, N.

3-(Acridinylamino)-5-(ethoxycarbonylamino)benzoic acid (14). Ethyl chloroformate (0.42 mL; 4.4 mmol) was added dropwise to a suspension of **13** (1.2 g, 3.6 mmol) in dry DMF (30 mL) containing pyridine (0.58 mL, 7.2 mmol) cooled in an ice bath and the mixture was stirred for 3 h. The thin-layer chromatography (SiO_2, $CHCl_3$/MeOH, 3:2 v/v) showed two products with R_f values of 0.9 (minor) and 0.5 (major). The reaction mixture was evaporated in vacuo to dryness and the solid residue was treated with 2% NH_4OH with vigorous stirring at room temperature for 40 min. After the mixture being filtered, the filter cake was triturated with a mixture of EtOH/MeOH/$CHCl_3$ (v/v/v 3/3/1) and the insoluble product was collected by filtration, washed with EtOH and dried to give crude **14**, 1.32 g (90%): mp >280 °C. Compound **14** was insoluble in most solvent and used directly for the next reaction without further purification. The crude product was used for ^{1}H NMR analysis. ^{1}H NMR (DMSO-d_6) δ 1.24 (3H, t, J=7.0 Hz, Me), 4.13 (2H, q, J=7.0 Hz, CH_2), 6.92 (1H, m, ArH), 7.15 (1H, m, ArH), 7.36 (2H, m, 2×ArH), 7.52 (1H, m, ArH), 7.78 (2H, m, 2×ArH), 8.15 (3H, m, 3×ArH), 9.76 and 11.05 (each 1H, brs, 2×NH).

3-(Acridinylamino)-5-(ethoxycarbonylamino)-*N*-(2-dimethylaminoethyl)-benzamide hydrochloride (15). *N*,*N*-Dimethylethylenediamine (0.15 mL, 1.38 mmol) was added to a mixture of **14** (500 mg, 1.25 mmol), Hünig's base (0.24 mL), and PyBOP (716 mg, 1.4 mmol) in dried DMF (30 mL). The reaction mixture was stirred in an ice bath for 2 h and then evaporated under reduced pressure to dryness. The crude product was purified by a column chromatography using $CHCl_3$/MeOH (4:1 v/v) as the eluant to give **15**, 550 mg (92%): mp 216–217 °C (EtOH); ^{1}H NMR (DMSO-d_6) δ 1.24 (3H, t, J=7.0 Hz, Me), 2.75 and 2.76 (each 3H, NMe_2) 3.18 (2H, d, J=5.2 Hz, CH_2), 3.55 (2H, d, J=5.2 Hz, CH_2), 4.13 (2H, q, J=7.0, CH_2), 7.49 (3H, m, ArH), 7.75 (1H, m, ArH), 7.79–8.08 (4H, m, 4×ArH), 8.25 (2H, m, 2×ArH), 8.69 (1H, m, ArH), 10.08 and 11.55 (each 1H, brs, 2×NH). Anal. ($C_{27}H_{29}N_5O_3$·2HCl·1.5H_2O) C, H, N.

Compound **16** was prepared in a similar manner from **14** and 3-dimethylamino-propylamine.

5-(9-Acridinylamino)-*o*-toluidine hydrochloride (AOT, 26a). A suspension of **25a** (3.31 g, 10.0 mmol) and 5% Pd/C (1.8 g) in MeOH (250 mL) containing concd HCl (6.0 mL) was hydrogenated at 50 psi for 2 h. The reaction mixture was filtered through a pad of Celite and washed well with MeOH. The combined filtrate and washings were evaporated in vacuo to dryness. The solid product was recrystallized from MeOH to give **26a**, 2.022 g (74%): mp 256–257 °C; ^{1}H NMR (DMSO-d_6) δ 2.29 (3H, s, Me), 6.92 (1H, m, ArH), 7.23 (1H, m, ArH), 7.45–7.49 (2H, m, ArH), 7.99–8.03 (2H, m, ArH), 8.10–8.12 (2H, m, ArH), 8.29–8.31 (2H, m, ArH), 11.56 (3H, brs, NH and NH_2). Anal. ($C_{20}H_{17}N_3$·HCl·4H_2O) C, H, N.

Following the same procedure as that for the synthesis of **22a**, compounds **26b**,**c** were prepared from **25b**,**c**, respectively.

AMT-ethylcarbamate hydrochloride (22a). Ethyl chloroformate (0.23 mL; 2.4 mmol) was added dropwise to a suspension of **21a** (672 mg, 2.0 mmol) in dry DMF (20 mL) containing pyridine (0.32 mL, 4.0 mol) at −10 °C. After the reaction mixture was stirred for 2 h and warmed up to the room temperature, it was evaporated in vacuo to dryness. The residue was treated with 1N HCl/EtOH in an ice bath to pH 2 and evaporated under reduced pressure to dryness. The solid residue was recrystallized from EtOH to give **22a**, 628 mg (77%); mp 226–227 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (3H, t, J=6.9 Hz, Me), 2.25 (3H, s, Me), 4.10 (2H, q, J=6.9 Hz, CH_2), 6.86 (1H, s, ArH), 7.30 (1H, s, ArH), 7.44 (1H, m, ArH), 7.48 (2H, m, 2×ArH), 8.01 (2H, m, 2×ArH), 8.12 (2H, m, 2×ArH), 8.27 (2H, m, 2×ArH), 9.87 and 11.49 (each 1H, brs, 2×NH). Anal. ($C_{23}H_{21}N_3O_2$·HCl·1.5H_2O) C, H, N.

Following the same procedure as that for the synthesis of **22a**, compounds **22b**,**c**, **24a–c**, **27a–c**, and **29a**,**b** were prepared from **21b**,**c**, **23a–c**, **26a–c**, and **28a**,**b**, respectively.

Biological assays

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), in a 72-h incubation, by XTT-tetrazolium assay, as described by Scudiero et al.[23] 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, USA). 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,[24,25] using a computer program on an IBM-PC workstation.[26]

Inhibition of topoisomerase II catalytic activity by drugs. Topo-II catalytic activity was assayed by the ATP-dependent relaxation of pBR322 supercoiled DNA.[27] Various concentrations of the drugs were incubated with 2 units of DNA topoisomerase II (Topogen) and 0.25 μg pBR322 DNA. The inhibition of the relaxation activity is determined by comparison with an untreated control. AHMA was used as a positive control.

Stimulation of DNA cleavage by Topo II. The method for evaluation of Topo II-induced double-stranded DNA breaks was described previously.[28] Linear pBR322 DNA was 3-end labeled, as described.[14] The reaction was performed at 37 °C for 15 min and stopped by the addition of 5 μL of 5% SDS and 0.75 mg/mL of proteinase K followed by incubation for 30 min at 50 °C. Samples were incubated with 5 μL of sample buffer

(50 mM EDTA, 50% (v/v) sucrose, and bromophenol blue) prior to loading onto the gel. After electrophoresis, the gel was dried and autoradiographed for 24 h.

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 the 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 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.[29]

DNA unwinding measurement. The DNA unwinding effect of drugs was measured using DNA circle-ligation assay as described previously.[30,31] Briefly, plasmid DNA was linearized with *HindIII* restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixture containing 66 mM Tris–HCl (pH 7.6), 6 mM $MgCl_2$, 10 mM dithiotreitol, 0.7 mM ATP, 0.6 μg of DNA and drugs were equilibrated at 15 °C for 10 min and than incubated with excess of T4 DNA ligase at 15 °C for 60 min. The reaction was stopped by addition of 20 mM EDTA. DNA was analyzed by agarose gel electrophoresis after removal the drugs from the reaction mixture: extraction with phenol and ether and precipitation with ethanol.

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 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) using 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.

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