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## Novel DNA-directed alkylating agents: Design, synthesis and potent antitumor effect of phenyl N-mustard-9-anilinoacridine conjugates via a carbamate or carbonate linker

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## ABSTRACT

A series of phenyl N-mustard-9-anilinoacridine conjugates via a carbamate or carbonate linker was synthesized for antitumor evaluation. The carbamate or carbonate linker is able to lower the reactivity of the phenyl N-mustard pharmacophore and thus, these conjugates are rather chemically stable. The *in vitro* studies revealed that these derivatives possessed significant cytotoxicity with IC<sub>50</sub> in sub-micromolar range in inhibiting human lymphoblastic leukemia (CCRF-CEM), breast carcinoma (MX-1), colon carcinoma (HCT-116) and human non-small cell lung cancer (H1299) cell growth *in vitro*. Compounds **10a**, **10b**, **10e**, **10i**, and **15a** were selected for evaluating their antitumor activity in nude mice bearing MX-1 and HCT-116 xenografts. Remarkably, total tumor remission was achieved by these agents with only one cycle of treatment. Interestingly, no tumor relapse was found in mice treated with **10a** over 129 days. This agent is capable of inducing DNA interstrand cross-linking in human non-small lung cancer H1299 cells in a dose dependent manner by modified comet assay and has a long half-life in rat plasma.

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## 1. Introduction

Using DNA alkylating N-mustards (e.g., chlorambucil and melphalan) for chemotherapy have a number drawbacks including (1) high chemical reactivity resulting in loss of drug's therapeutic efficacy by reacting with other cellular nucleophiles such as proteins and low molecular weight thiols (inducing cellular resistance by increased levels of glutathione);<sup>1–3</sup> (2) lack of intrinsic DNA binding affinity of the alkylating pharmacophore (High ratio of genotoxic mono-adducts to cross-links (20:1), carcinogenicity or bone marrow toxicity);<sup>4</sup> and (3) reducing cytotoxicity via a DNA repair mechanism.<sup>5</sup> To overcome these drawbacks, one of the strategies is to design DNA-directed alkylating agents by linking the alkylating pharmacophore with DNA-affinic molecules (such as acridines, anthraquinones, quinolines, phenanthridines, or 9-anilinoacridines).<sup>6–11</sup> It has been demonstrated that these conjugates are able to modify the pattern of DNA alkylation and possess high-

er cytotoxicity and potency than their corresponding untargeted alkylating agents. Another approach to minimize these drawbacks is to prepare prodrug. Early reports showed that prodrug cyclophosphamide exhibited improved pharmacokinetic and has long half-life in animal model.<sup>12</sup> Apart from cyclophosphamide and the related derivatives, Springer et al. have synthesized aniline or phenyl mustards linked to L-glutamic acid moiety through a urea (**1**, Z = NH, Chart 1), carbamate (**1**, Z = O)<sup>13</sup> or carboxamide (**2**, CMDA)<sup>14,15</sup> linker for antibody-directed enzyme prodrug therapy (ADEPT). The enzymatic hydrolysis by cyclopeptidase (CPG2) of these prodrugs leads to release the active N-mustard pharmacophore at the tumor site. Prodrugs, **3**<sup>16</sup>, **4**<sup>17</sup> and **5**<sup>18</sup> were also prepared for melanocyte directed enzyme prodrug therapy (MDEPT). The reactivation of these prodrugs requires enzymatic hydrolysis by tyrosinase. These studies demonstrated that the urea, carbamate, or carboxamide linker is capable of lowering the reactivity of aniline or phenol N-mustard pharmacophore resulting in formation of rather stable N-mustard derivatives.

One of our ongoing projects on the research and development of new potential antitumor agents, we focus on designing and developing DNA-directed alkylating agents. We have previously synthe-

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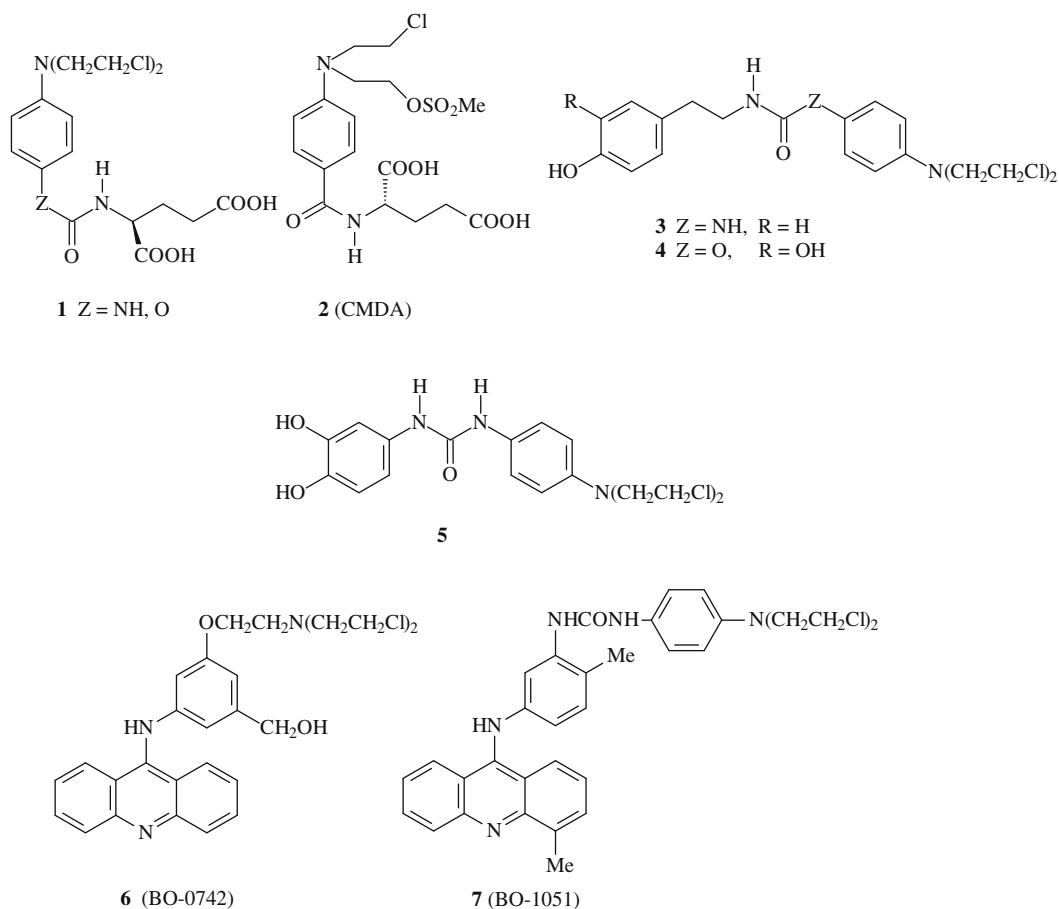


Chart 1.

sized a series of alkyl N-mustards linked to DNA-affinic molecules (e.g., 9-anilinoacridines or acridine). The N-mustard pharmacophore was linked to the aniline ring or acridine chromophore of 9-anilinoacridines with alkoxy (O-C<sub>1-4</sub>) or alkyl (C<sub>1</sub>) linker.<sup>19–21</sup> It revealed that these agents exhibited potent antitumor effects in inhibiting various human acute lymphoblastic leukemia cells (CCRF-CEM) and solid tumors in cell culture.<sup>20</sup> Among these agents, compound **6** (BO-0742) was found to have potent therapeutic efficacy against human breast MX-1 and ovarian SK-VO3 xenografts in animal model. However, our unpublished results showed that BO-0742 has a short half-life (<25 min) in mice. To improve the poor pharmacokinetics of BO-0742, we have recently synthesized phenyl N-mustard-9-anilinoacridine conjugates via a urea linker.<sup>22</sup> It was shown that these conjugates exhibited a broad spectrum of antitumor activity. Of these agents, BO-1051 (**7**) possessed potent therapeutic efficacy against human tumor xenografts in nude mice. Total tumor remission (or complete remission, CR) was achieved in nude mice bearing human breast MX-1 xenograft and showed no relapse over 70 days. Human glioma U87 MG in nude mice was also significantly suppressed. We also reported that this agent is capable of inducing DNA interstrand cross-linking. Notably, BO-1051 was found to be more stable than BO-0742 in intravenous injection vehicle and in rat plasma.

From these studies, it clearly suggested that the selection of N-mustard (alkyl or phenyl N-mustard), DNA-affinic molecule and linker are important since they may affect the antitumor activity, DNA-drug interaction and stability of these DNA-directed alkylating agents. In general, alkyl N-mustards are very reactive and unstable due to the inductive effect of alkyl function leading to rapid generation of the reactive aziridinium cation intermediate, which readily interacts with DNA forming interstrand cross-linking.

While, the reactivity of phenyl N-mustards is relatively weaker than alkyl N-mustards due to the electron-withdrawing property of the phenyl ring. Earlier works on the development of DNA-directed alkylating agents have demonstrated that these agents have distorted patterns of DNA alkylation by the attached DNA-affinic molecules.<sup>23</sup> In our studies,<sup>19–22</sup> we utilized 9-anilinoacridines such as 3-(9-acridinylamino)-5-hydroxymethylanilines (AHMAs),<sup>24</sup> 3-(9-acridinylamino)toluidines (ATs),<sup>25</sup> 3-(9-acridinylamino)anisidines (AAs),<sup>26</sup> and AHMA-alkylcarbamates<sup>27</sup> previously synthesized in our laboratory as the DNA-affinic molecules for linking with N-mustard residue.<sup>19–22</sup> As a result, these N-mustard-9-anilinoacridine conjugates were found to have significantly improved cytotoxicity and potent therapeutic efficacy against various human tumor xenografts suggesting that these DNA-intercalating agents are very useful as DNA-affinic molecules (or carriers) for designing DNA-directed alkylating agents. As for the spacer used for linking N-mustard pharmacophore with DNA-affinic molecules, the length and types of linker are also critical since they may also influence both the DNA-drug interaction capability and chemical stability. The advantage of using the urea, carbamate, or carboxamide linker is that they are able to stabilize the reactive phenyl N-mustards as described previously. Based on these findings, we may generate new and potent DNA-directed alkylating agents having improved bioavailability and therapeutic efficacy.

To continue our research on developing DNA-directed alkylating agents, we have synthesized a series of new N-mustards-9-anilinoacridin conjugates using the carbamate or carbonate groups as a spacer to realize whether these conjugates have improved antitumor profiles and chemical stability. The phenol N-mustard residue was attached to the amino function of the aniline ring of AHMAs, ATs and AAs via a carbamate spacer. We also coupled the aniline

or phenol N-mustard residue with the hydroxyl substituent on the aniline ring of AHMA-alkylcarbamates (**11a–c**) via a carbamate or carbonate linker. The results showed that they exhibited promising antitumor activity against various human tumor xenografts. In this paper we describe the synthesis, antitumor effect, DNA interstrand cross-linking and the stability in plasma of the new N-mustard derivatives.

## 2. Chemistry

9-Anilinoacridines such as AHMAs (**8a,b**),<sup>24</sup> ATs (**8c,d,e,f**),<sup>25</sup> AAs (**8g,h,i,j,k**),<sup>26</sup> and AHMA-alkylcarbamates (**11a–c**)<sup>27</sup> were synthesized by the methods previously developed in our laboratory. These agents were used as the DNA-directed carriers for constructing the new N-mustard-9-anilinoacridine conjugates. The key reagents, 4-(bis(2-chloroethyl)amino)phenyl 4-nitrophenyl carbonate (**9**),<sup>17,28,29</sup> *N,N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**12**),<sup>16</sup> and 4-[*N,N*-bis(2-chloroethyl)-amino]phenylisocyanate (**13**)<sup>30</sup> were prepared by following the literature methods. The synthesis of N-mustards linked to the amino function of aniline ring of 9-anilinoacridine conjugates via a carbamate linker (**10a–k**) is shown in Scheme 1. Reaction of 9-anilinoacridine (**8a–k**) with **9** in dry DMF in the presence of pyridine at room temperature afforded **10a–k** in a fair to good yield (Table 1). The preparation of N-mustards linked to the hydroxymethyl function of AHMA-alkylcarbamate (**11a–c**) via a carbamate or carbonate linkage is shown in Scheme 2. The freshly prepared **13** (generated from **12** by treating with triphosgene) in anhydrous DMF in presence of TEA gave the corresponding N-mustard-AHMA-alkylcarbamate conjugates having a carbamate spacer (**14a–c**) in moderate yields (Table 1). While, the coupling of **11a–c** with **9** to prepare N-mustard-AHMA-alkylcarbamate conjugates bearing a carbonate linker was carried out in anhydrous DMF in presence of pyridine/DMAP at room temperature. However, only **15a** was obtained in 25% after purification by liquid flash column chromatography. Attempts to prepare **15b,c** under various reaction conditions were failed; a mixture of complex decomposition products was observed by silica gel thin-layer chromatography.

## 3. Biological results and discussion

### 3.1. In vitro cytotoxicity

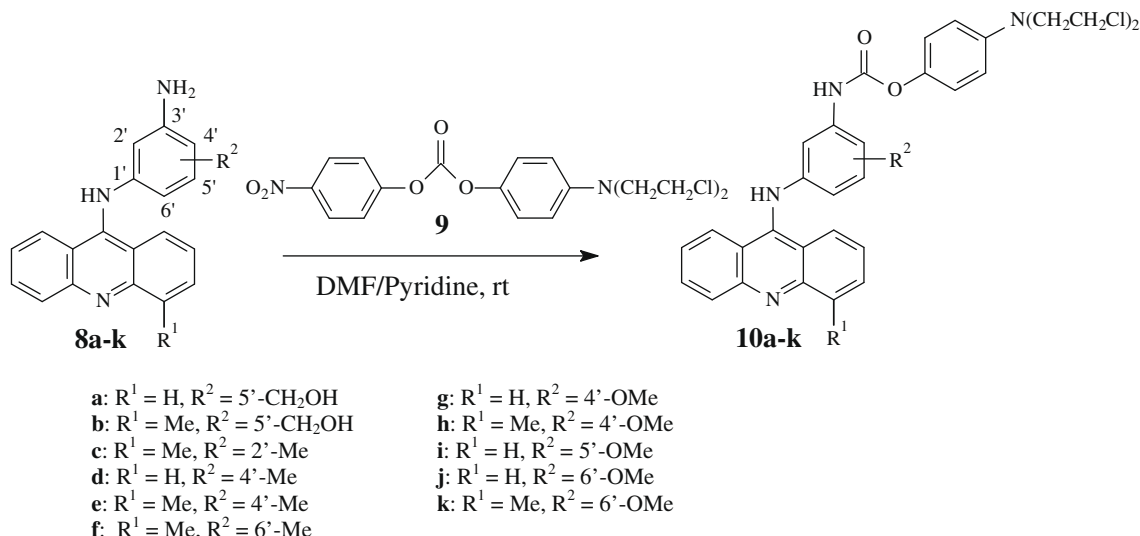
Table 2 shows the antiproliferative activities of the newly synthesized N-mustard-9-anilinoacridine conjugates (**10a–k**, **14a–c**

**Table 1**

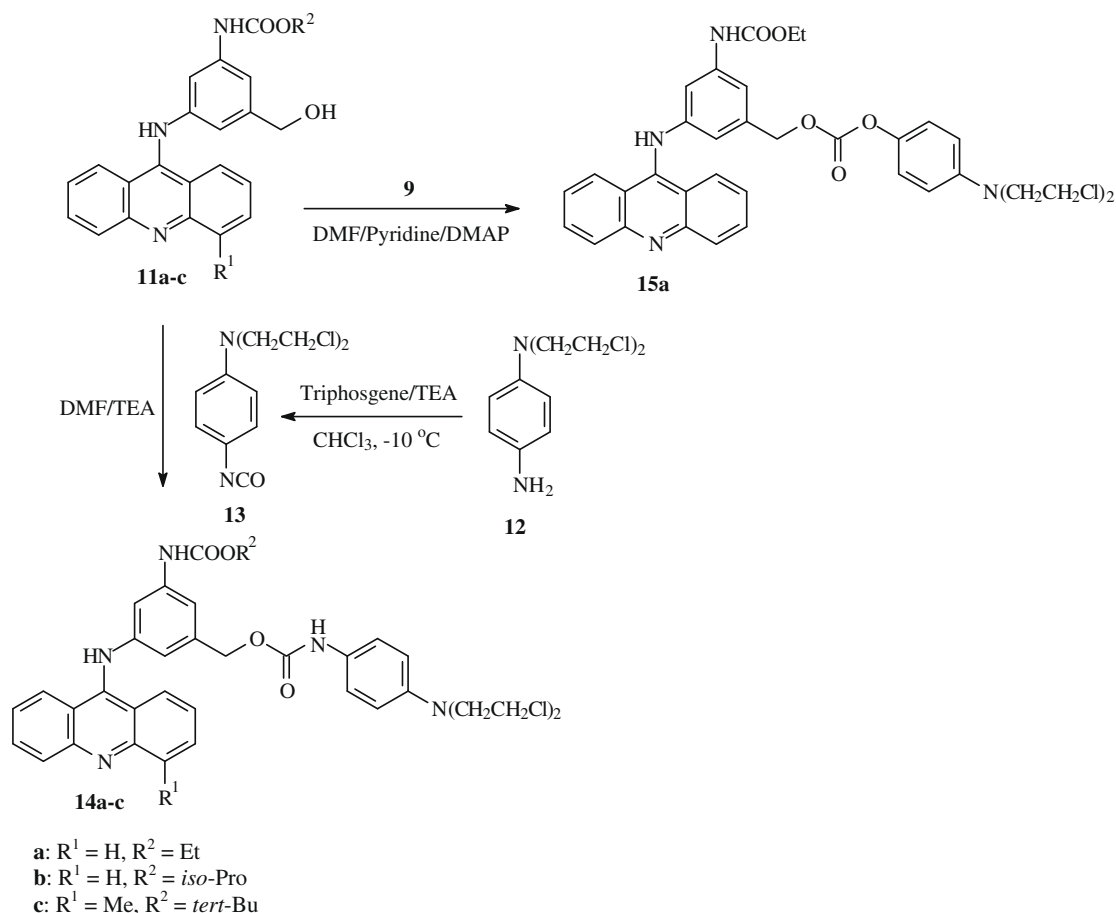
Analytical data and yields of new N-mustards conjugate (**10a–k**, **14a–c** and **15a**)

Compd.	Mp (°C)	Yield (%)	Molecular formula	Analysis
<b>10a</b>	241–242	79	C <sub>31</sub> H <sub>28</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10b</b>	240–242	61	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10c</b>	202–203	69	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>2</sub>	C, H, N
<b>10d</b>	138–139	59	C <sub>31</sub> H <sub>28</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>2</sub>	C, H, N
<b>10e</b>	154–156	52	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>2</sub>	C, H, N
<b>10f</b>	104–105	49	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>2</sub>	C, H, N
<b>10g</b>	190–192	63	C <sub>31</sub> H <sub>28</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10h</b>	195–196	47	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10i</b>	179–180	21	C <sub>31</sub> H <sub>28</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10j</b>	194–195	78	C <sub>31</sub> H <sub>28</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>10k</b>	186–188	52	C <sub>32</sub> H <sub>30</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub>	C, H, N
<b>14a</b>	178–179	60	C <sub>34</sub> H <sub>33</sub> Cl <sub>2</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>14b</b>	120–122	13	C <sub>36</sub> H <sub>37</sub> Cl <sub>2</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>14c</b>	228–230	34	C <sub>37</sub> H <sub>39</sub> Cl <sub>2</sub> N <sub>5</sub> O <sub>4</sub>	C, H, N
<b>15a</b>	131–133	25	C <sub>34</sub> H <sub>32</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>5</sub>	C, H, N

and **15a**) against human lymphoblastic leukemia (CCRF-CEM) cell growth in vitro. It demonstrated that the newly synthesized conjugates possess significant cytotoxicity with IC<sub>50</sub> in submicro molar range. The structure-activity relationship (SAR) studies of these agents against the CCRF-CEM cell growth revealed that in ATs series, C4'-Me substituted compound (**10d**) was more cytotoxic than the corresponding C2'-Me (**10c**) or C6'-Me (**10f**). In the AAs series, the C4'-OMe derivatives (**10g** and **10h**) were about 3–20-fold more potent than the C5'-OMe (**10i**) or C6'-OMe (**10j** and **10k**) derivatives. It is interesting to note that in the series of AA conjugates, the cytotoxicity was increased by adding the methyl group at C4 position of the acridine ring (**10g** vs **10h**, **10j** vs **10k**). In contrast, the C4-Me substituted compound (**10e**) was less active than the C4-unsubstituted derivative (**10d**). The N-mustard derivatives bearing CH<sub>2</sub>OH substituent (**10a** and **10b**) were as potent as C4'-Me (**10d**) and C4'-OMe (**10g**) derivatives. Of these derivatives, **10h** (C4'-OMe-C4-Me) was the most cytotoxic with the IC<sub>50</sub> value of 0.024 μM. The SAR study of the N-mustard-AHMA-alkylcarbamate conjugates (**14a–c**) showed that AHMA-ethylcarbamate (**14a**) was more potent than the corresponding *iso*-propyl and *tert*-butylcarbamates (**14b** and **14c**). In comparison of the cytotoxicity of the conjugates having carbamate and carbonate linker (**14a** vs **15a**, respectively), **14a** was more cytotoxic than **15a**. Table 2 also shows that the newly synthesized conjugates are more cytotoxic than AHMA (**8a**), but less potent than BO-0742 (**6**) suggesting that N-mustard masked derivatives are more cytotoxic than the corre-



**Scheme 1.** Synthesis of N-mustards linked to the amino function of 9-anilinoacridines (**8a–k**) via a carbamate linker.



**Scheme 2.** Synthesis of N-mustards linked to the hydroxymethyl function of AHMA-alkylcarbamate (**11a–c**) via a carbamate or carbonate linker.

**Table 2**

Cytotoxicity of new N-mustard conjugates, **10a–k**, **14a–c** and **15a** against human lymphoblastic leukemia (CCRF-CEM) and its drug-resistant sublines (CCRF-CEM/Taxol and CCRF-CEM/VBL) and solid tumors (MX-1 and HCT-116) cell growth *in vitro*<sup>a</sup>

Compound	IC <sub>50</sub> (μM)				
	CCRF-CEM	CCRF-CEM/Taxol <sup>b</sup>	CCRF-CEM/VBL <sup>b</sup>	MX-1	HCT-116
<b>10a</b>	0.075 ± 0.016	0.140 ± 0.032[1.9×] <sup>c</sup>	0.184 ± 0.028[2.5×]	0.099 ± 0.004	0.568 ± 0.037
<b>10b</b>	0.067 ± 0.013	0.097 ± 0.018[1.4×]	0.127 ± 0.019[1.9×]	0.084 ± 0.003	0.438 ± 0.036
<b>10c</b>	0.16 ± 0.026	0.174 ± 0.004[1.09×]	0.142 ± 0.003[0.89×]	0.181 ± 0.008	0.320 ± 0.010
<b>10d</b>	0.076 ± 0.014	0.216 ± 0.004[2.8×]	0.158 ± 0.006[2.1×]	0.214 ± 0.004	0.434 ± 0.021
<b>10e</b>	0.132 ± 0.016	0.169 ± 0.011[1.3×]	0.157 ± 0.011[1.2×]	0.286 ± 0.025	0.607 ± 0.038
<b>10f</b>	0.132 ± 0.053	0.138 ± 0.062[1.05×]	0.093 ± 0.014[0.7×]	0.163 ± 0.014	0.263 ± 0.055
<b>10g</b>	0.085 ± 0.034	0.16 ± 0.042[1.9×]	0.119 ± 0.013[1.4×]	0.181 ± 0.005	0.186 ± 0.003
<b>10h</b>	0.024 ± 0.013	0.027 ± 0.006[1.1×]	0.19 ± 0.001[7.9×]	0.138 ± 0.012	0.238 ± 0.006
<b>10i</b>	0.200 ± 0.002	0.309 ± 0.003[1.5×]	0.108 ± 0.001[0.54×]	0.340 ± 0.007	1.712 ± 0.012
<b>10j</b>	0.541 ± 0.025	0.583 ± 0.003[1.08×]	0.254 ± 0.004[0.14×]	1.555 ± 0.034	0.709 ± 0.017
<b>10k</b>	0.205 ± 0.052	0.210 ± 0.001[1.0×]	0.11 ± 0.004[0.54×]	0.338 ± 0.02	0.841 ± 0.044
<b>14a</b>	0.085 ± 0.001	0.243 ± 0.001[2.9×]	0.319 ± 0.006[3.9×]	0.597 ± 0.007	0.752 ± 0.009
<b>14b</b>	0.189 ± 0.047	0.648 ± 0.057[3.4×]	0.753 ± 0.022[4×]	1.13 ± 0.045	0.301 ± 0.053
<b>14c</b>	0.750 ± 0.17	3.358 ± 0.22[4.5×]	1.416 ± 0.018[1.9×]	5.933 ± 0.657	2.403 ± 0.106
<b>15a</b>	0.315 ± 0.004	0.509 ± 0.004[1.6×]	0.482 ± 0.004[1.5×]	0.089 ± 0.001	0.639 ± 0.005
<b>6</b> <sup>20</sup>	0.007	0.0075[1.1×]	0.0340[4.9×]	0.0035	0.0055
<b>8a</b> <sup>20</sup>	0.753	0.600[0.8×]	1.60[2.70×]	0.0035	ND <sup>d</sup>
Taxol	0.0031 ± 0.0001	0.429 ± 0.042[330×]	1.274 ± 0.052[980×]	0.046 ± 0.0007	0.0011 ± 0.0003
Vinblastine	0.00073 ± 0.0009	0.078 ± 0.011[106.2×]	0.496 ± 0.121[679.5×]	0.0022 ± 0.0002	0.0011 ± 0.0003

<sup>a</sup> Cell growth inhibition was measured by the XTT<sup>31</sup> assay for leukemic cells and the SRB assay<sup>32</sup> for solid tumor cells after 72 h incubation using a microplate spectrophotometer as described in experimental section. IC<sub>50</sub> values were determined in duplicate or triplicate from dose–effect relationship at six or seven concentrations of each drug by using the COMPUSYN software by Chou and Martin<sup>35</sup> based on the median-effect principle and plot and serial deletion analysis. Ranges given for Taxol and vinblastine were mean ± SE ( $n = 4$ ).

<sup>b</sup> CCRF-CEM/Taxol and CCRF-CEM/VBL are subcell lines of CCRF-CEM cells that are 330-fold resistant to Taxol, and 680-fold resistant to Vinblastine, respectively, when comparing with the IC<sub>50</sub> of the parent cell line.

<sup>c</sup> Numbers in the brackets are fold of cross-resistant determined by comparison with the corresponding IC<sub>50</sub> of the parent cell line.

<sup>d</sup> ND = not determined.

sponding parent compounds. Moreover, it also demonstrates that the alkyl N-mustard derivatives are more potent than the corresponding phenyl N-mustard counter part (**6** vs **10a**).

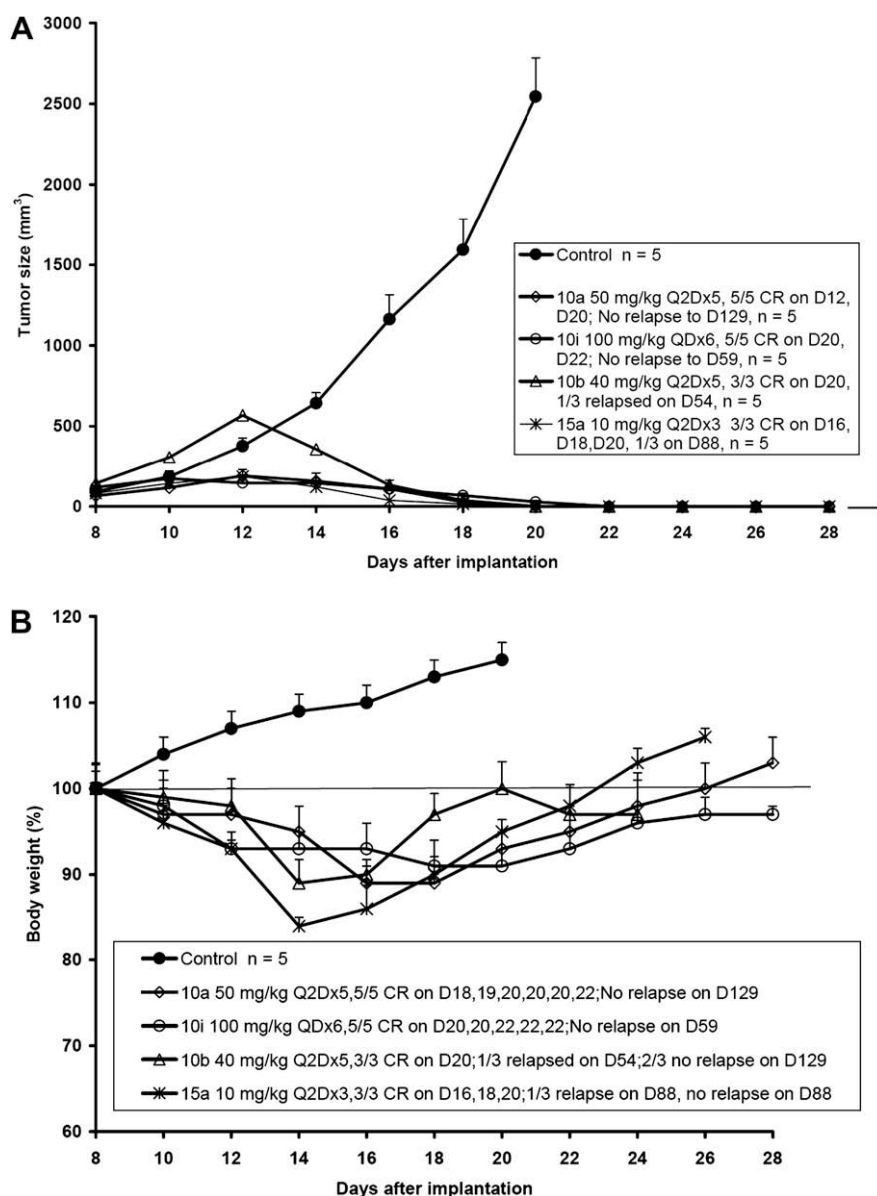
We have also evaluated the antiproliferative activities of new N-mustard conjugates (**10a–k**, **14a–c** and **15a**) against MX-1 and HCT-116 solid tumor cell growth (Table 2). In general, the position

**Table 3**  
Cytotoxicity of new N-mustard conjugates, **10a**, **10b**, **10f**, **10h**, **14b** and **15a** against non-small cell lung cancer (H1299), human prostate cancer (PC3), human lung adenocarcinoma (CL1-0 and CL1-5) and human breast adenocarcinoma (MCF-7) cell growth in vitro<sup>a</sup>

Compound	IC <sub>50</sub> (μM) <sup>b</sup>				
	H1299	PC3	CL1-0	CL1-5	MCF-7
<b>10a</b>	0.284 ± 0.083	1.609 ± 0.340	2.695 ± 0.773	5.064 ± 1.459	4.797 ± 0.531
<b>10b</b>	0.457 ± 0.040	5.977 ± 1.912	1.869 ± 0.789	12.648 ± 1.723	9.01 ± 0.918
<b>10f</b>	0.623 ± 0.023	4.469 ± 1.796	4.657 ± 1.432	10.588 ± 2.476	5.713 ± 0.972
<b>10h</b>	0.357 ± 0.043	1.576 ± 0.585	2.251 ± 0.806	8.85 ± 1.28	3.433 ± 0.991
<b>14b</b>	9.171 ± 0.195	15.980 ± 4.062	3.996 ± 0.507	6.629 ± 1.352	7.012 ± 0.773
<b>15a</b>	0.527 ± 0.071	23.331 ± 4.974	0.410 ± 0.242	4.56 ± 3.313	19.802 ± 6.596
Cisplatin	4.707 ± 0.574	11.803 ± 5.775	56.917 ± 16.984	27.741 ± 3.913	19.872 ± 1.885

<sup>a</sup> Cell growth inhibition was measured by the WST-1 assay<sup>34</sup> after 72 h incubation using a microplate spectrophotometer as described in Section 5.

<sup>b</sup> The values are averages of at least two separate determinations.



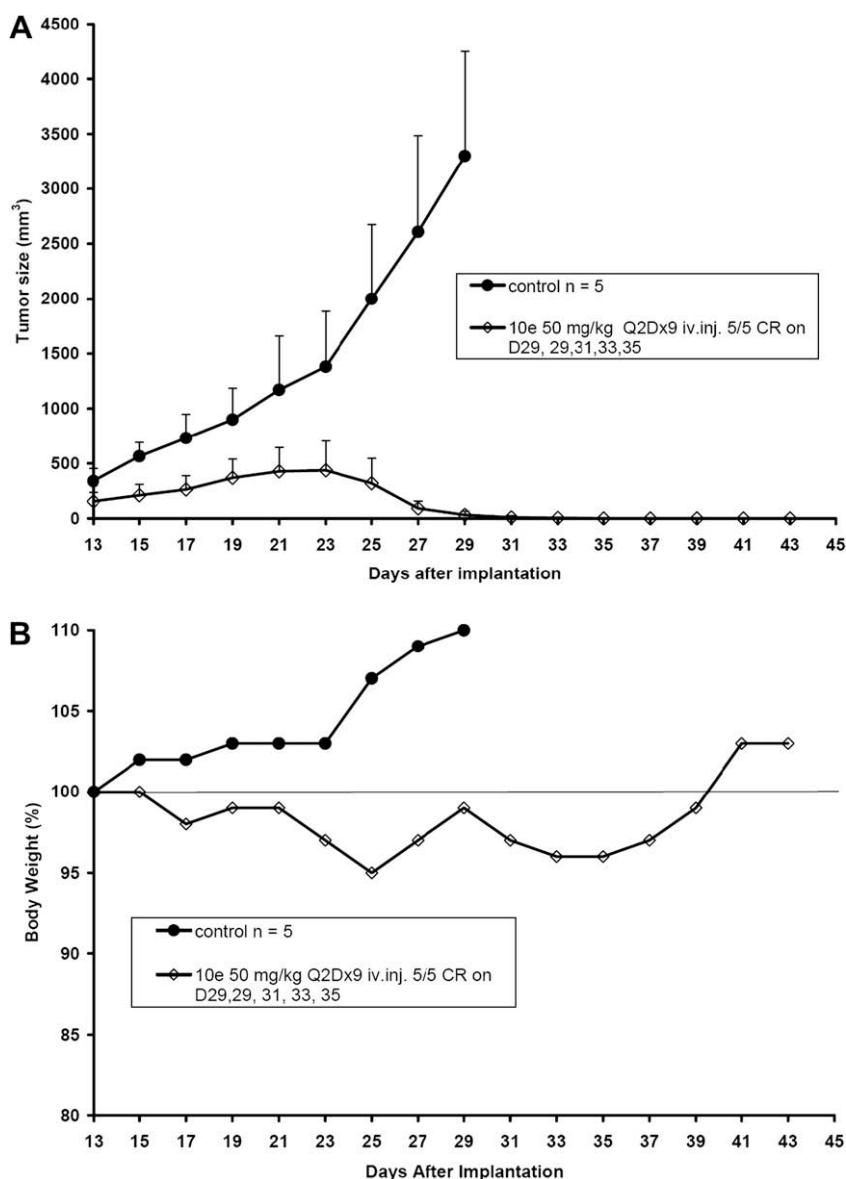
**Figure 1.** The therapeutic effects of **10a**, **10b**, **10i**, and **15a** in nude mice bearing MX-1 (iv injection,  $n = 5$ ). (A) For average tumor size changes;  $p$  values for **10a**, **10b**, **10i**, and **15a** are  $<0.0001$  (D14–20),  $<0.0008$  (D14–20),  $<0.0001$  (D16–18), and  $<0.0005$  (D16–18), respectively. (B) For average body weight changes.

of the N-mustard residue either linked to the amino function of AHMAs, ATs or AAs (**10a–k**) or to the hydroxymethyl of AHMA-alkylcarbamates (**14a–c** or **15a**) did not greatly affect their cytotoxicity against both cell lines. Among the new conjugates tested against MX-1 cell growth in vitro, **10a**, **10b**, and **15a** were shown to have significant cytotoxicity with  $IC_{50}$  values of 0.099, 0.084, and 0.089  $\mu$ M, respectively. In addition, all new conjugates (**10a–k**, **14a–c** and **15a**) possessed a comparable cytotoxicity against HCT-116 cell line. To explore further, compounds **10a**, **10b**, **10f**, **10h**, **14b** and **15a** were selected to study their cell growth inhibitory effect against other solid tumor such as non-small cell lung cancer (H1299), human prostate cancer (PC3), human lung adenocarcinoma (CL1-0), and CL1-5 and human breast adenocarcinoma (MCF-7) (Table 3). The SAR study revealed that these agents were more cytotoxic in inhibiting H1299 cell growth with  $IC_{50}$  value in sub micro molar range than PC3, CL1-0, CL1-5 or MCF-7 cell lines. Importantly, all the selected derivatives were about 1–140-fold more potent than the cisplatin against CL1-0, CL1-5 and MCF-7 cell growth. Among these agents, compound **10a** was the most potent in inhibiting H1299 cell growth with  $IC_{50}$  value of 0.284  $\mu$ M.

We used CCRF-CEM/Taxol and CCRF-CEM/VBL, which are CCRF-CEM sub-cell lines resistant to Taxol and Vinblastine, for comparison to determine whether the newly synthesized compounds are multi-drug resistant toward these two cell lines. As shown in Table 2, the newly synthesized derivatives have little or no cross-resistance to either taxol or vinblastine. This suggests that all new conjugates are neither a good substrate of membrane multi-drug resistance transporters (i.e., p-glycoprotein) nor mutated tubulin. Thus, the newly synthesized compounds are effective against multiple drug resistant tumors.

### 3.2. In vivo therapeutic effect

Compounds **10a**, **10b**, **10e**, **10i**, and **15a** were selected for evaluating their antitumor therapeutic efficacy in animal model which is based on their in vitro cytotoxicity, solubility and toxicity to the host. Figure 1 shows the therapeutic effects of **10a**, **10b**, **10i** and **15a** in nude mice bearing mammary MX-1 xenografts at the maximal tolerated doses [50 mg/kg, Q2D  $\times$  5; 40 mg/kg, Q2D  $\times$  5, 100 mg/kg, QD  $\times$  6 and 10 mg/kg, Q2D  $\times$  3, respectively, via intra-



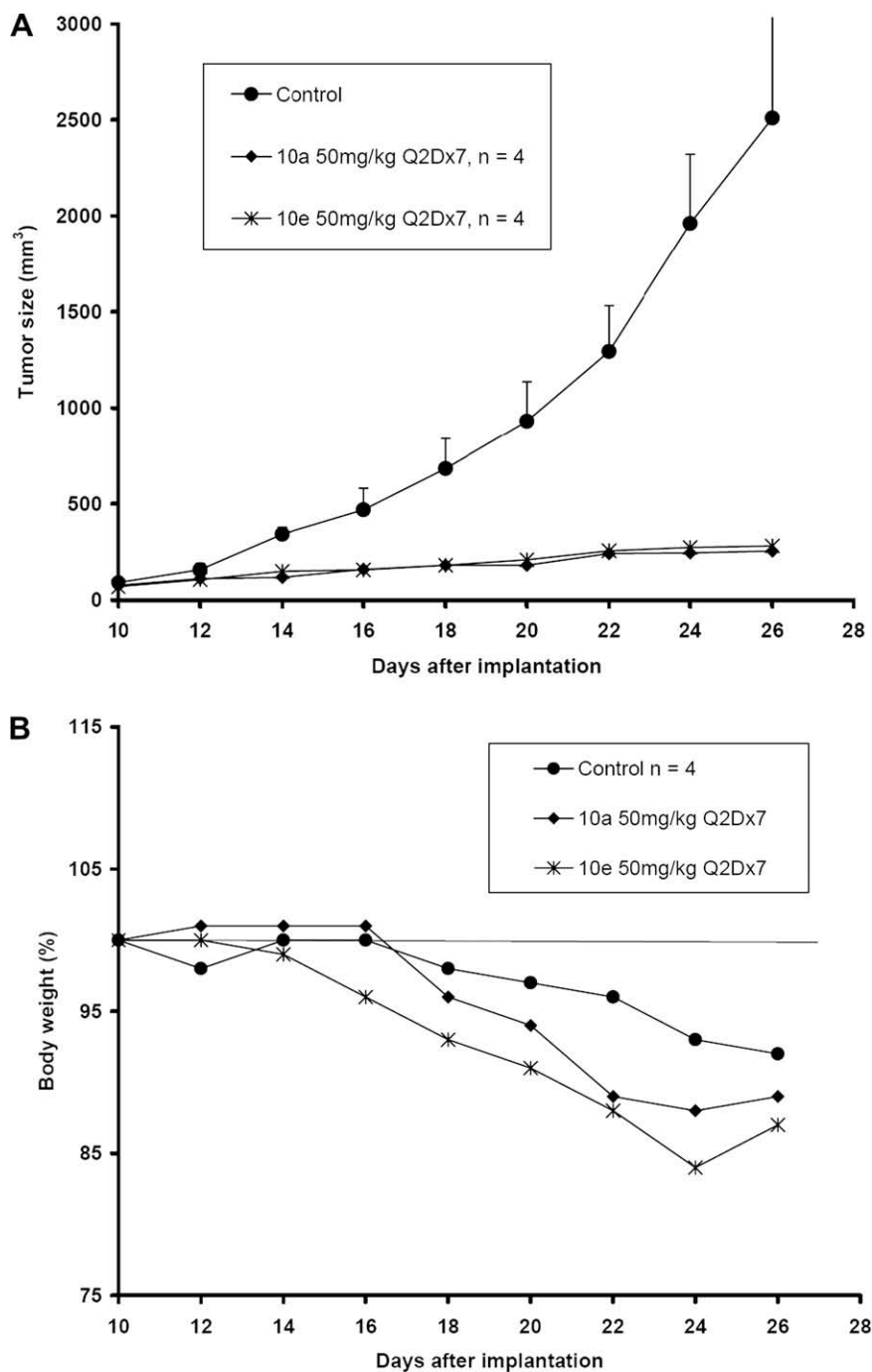
**Figure 2.** The therapeutic effects of **10e** in nude mice bearing MX-1 (iv injection,  $n = 5$ ). (A) For average tumor size changes;  $p$  value  $< 0.0001$  (D27–29); (B) for average body weight changes.

venous injection (iv injection)]. Remarkably, in all cases, total tumor remission (or complete remission, CR) was achieved with only one cycle treatment (Fig. 1A). No tumor relapse was found in mice treated with **10a** and partial mice treated with **10b** on Day 129 of experimentation. Compound **10e** also led to complete tumor remission at the dose of 50 mg/kg, Q2D  $\times$  9, iv injection. (Fig. 2A and B). Figure 3 shows the therapeutic effects of **10a** and **10e** in nude mice bearing colon HCT-116 xenografts. Compound **10a** and **10e** showed, at the doses of 50 and 50 mg/kg, respectively, significant tumor growth inhibition (98% and 89%, respectively) in comparison to untreated control. Although 8–15% body-weight change was found during maximal tolerated treatment (Fig. 1B, 2B, and 3B), the body-weight of mice was readily recovered after

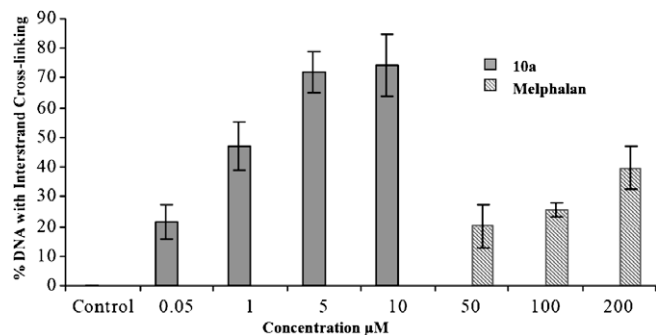
cession of treatment suggesting that the tested compounds have relatively low toxicity to the hosts.

### 3.3. DNA interstrand cross-linking study

To realize whether the newly synthesized conjugates are able to cross-link to DNA doubled strands, the single cell gel electrophoresis assay (modified comet assay)<sup>28</sup> was employed to determine the level of DNA interstrand cross-linking. The representative compound **10a** was subjected to DNA cross-linking studies in human non-small lung cancer H1299 cells by the modified comet assay and was compared with melphalan. The results showed that **10a** was capable of inducing DNA interstrand cross-linking in a dose-



**Figure 3.** The therapeutic effects of **10a** and **10e** in nude mice bearing HCT-116 xenografts (iv injection,  $n = 4$ ). (A) For average tumor size changes;  $p$  value  $<0.0001$ ; (B) for average body weight changes.



**Figure 4.** DNA cross-linking in H1299 cells measured by the modified comet assay. Results were generated as described in Section 5. The experiments were performed in duplicate and repeated three times. Data points represent the mean  $\pm$  SE of each test condition.

dependent manner (Fig. 4). At the dose of 10  $\mu$ M, this agent induced 74.2% DNA cross-linking, while, melphalan induced 39.7% DNA cross-linking at the dose of 200  $\mu$ M under the same experimental conditions. Figure 5 shows the distribution of tail moment in H1299 cells. The production of an essentially random distribution of single-strand breaks is obtained by means of 20 Gy X rays. As cross-linking agents connect DNA with DNA and/or histone proteins, they decreased DNA migration in the comet assay. The comet images clearly show the concentration-dependent reduction of DNA migration in all H1299 cells with increasing concentrations of the cross-linker **10a**. The results suggested that DNA interstrand cross-linking may be the main mechanism of action of **10a** and the related compounds.

### 3.4. Stability in rat plasma

Compound **10a** was further selected to study its chemical stability in rat plasma. The degradation of this agent was detected by HPLC. The detection limit is 20 ng/ml of the authentic **10a** in the rat plasma. It revealed that **10a** is a stable N-mustard derivative in rat plasma with long half-life ( $t_{1/2} = 6.41 \pm 0.86$  h,  $n = 3$ ). These results demonstrate that the newly prepared N-mustards are chemically and metabolically stable derivatives.

## 4. Conclusion

In the current studies, we have designed and synthesized a series of DNA-directed alkylating agents by linking the phenyl N-mustard pharmacophore with the amino function of the aniline ring of various 9-anilinoacridine derivatives via a carbamate linker (**10a–k**) or

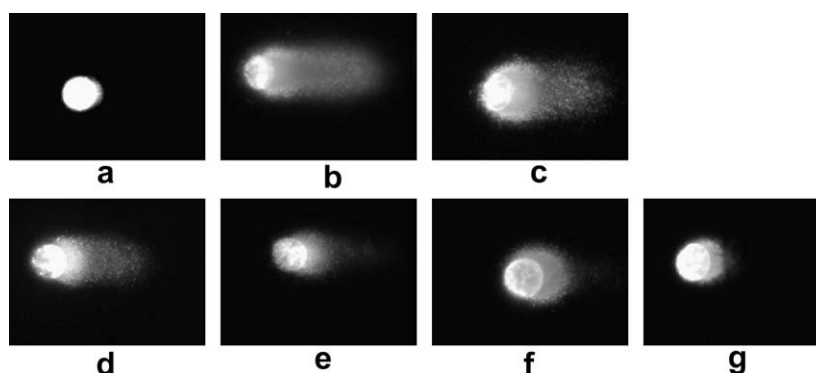
to the hydroxymethyl function of AHMA-alkylcarbamates via a carbamate or carbonate spacer (**14a–c** and **15a**, respectively). The SAR studies showed that, compounds **10a–k** are more cytotoxic or as potent as **14a–c**. The antitumor therapeutic efficacy studies against human tumor xenografts demonstrated that the newly synthesized conjugates exhibited potent antitumor efficacy against breast carcinoma MX-1 and colon carcinoma HCT-116 xenografts. For MX-1 xenograft, complete tumor remission was achieved after treatment of **10a**, **10b**, **10e**, **10i**, and **15a** and maintain no relapse on Day 129 by **10a** and **10b**. For HCT-116 xenografts, tumor growth inhibition was 98% and 89% by **10a** and **10e**, respectively. Moreover, we also found that **10a** is able to induce DNA interstrand cross-linking and the DNA cross-linking capability is much more effective than that of melphalan. In our recent studies, we demonstrated that urea linker in phenyl N-mustard-9-anilinoacridine conjugates plays a very important role, especially for stabilizing the reactive N-mustard residue. The current studies also show that the carbamate or carbonate linkers play an important role in enhancing drug's therapeutic effects, DNA interstrand cross-linking and stability.

## 5. 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 and 230–400 mesh). Thin-layer chromatography was performed on Silica Gel G60 F<sub>254</sub> with short-wavelength UV light for visualization. Elemental analysis was done on a Heraeus CHN-O Rapid instrument. <sup>1</sup>H NMR spectra were recorded on 400 MHz spectrometers. The chemical shifts were reported in ppm ( $\delta$ ) relative to TMS.

### 5.1. General procedure for the preparation of N-mustard linked to the amino function of 9-anilinoacridines via a carbamic acid ester linkage (**10a–k**)

9-Anilinoacridines such as AHMAs (**8a,b**),<sup>24</sup> ATs (**8c,d,e,f**),<sup>25</sup> AAs (**8g,h,i,j,k**),<sup>26</sup> and AHMA-alkylcarbamates (**11a–c**)<sup>27</sup> were synthesized by the methods previously developed in our laboratory. These agents were used as the DNA-directed carriers for constructing the N-mustard-9-anilinoacridine conjugates. The key intermediate, 4-[bis-(2-chloroethyl)amino] phenyl ester 4-nitro-phenyl ester (**9**),<sup>17,28,29</sup> *N,N*-Bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**12**),<sup>16</sup> and 4-[*N,N*-bis(2-chloroethyl)amino]phenyl-isocyanate (**13**)<sup>30</sup> were prepared by following the literature methods. The reaction of **9** with appropriate 9-anilinoacridines (**8a–k**)<sup>24–26</sup> was carried out in dry DMF in the presence of pyridine to give **10a–k**. The final products were purified either by recrystallization from an appropriate solvent or by column chromatography using



**Figure 5.** Typical comet images from H1299 cell treated ex vivo with **10a** and melphalan. The presence of cross-links retards the electrophoretic mobility of alkaline denatured cellular DNA. Cross-links are, therefore, quantitated as the decrease in comet tail moment compared with irradiated control. (a) Control; (b) 20 Gy irradiation; (c) melphalan (200  $\mu$ M); (d) **10a** 0.05  $\mu$ M; (e) **10a** 1  $\mu$ M; (f) **10a** 5  $\mu$ M; (g) **10a** 10  $\mu$ M.



(SiO<sub>2</sub>, CHCl<sub>3</sub>/MeOH, v/v 100:1). The detailed procedure was described as follows.

#### 5.1.1. [3-(Acridin-9-ylamino)-5-hydroxymethyl-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino]-phenyl ester (10a)

Compound **9** (1.123 g, 2.7 mmol) was added into a solution of [3-(acridin-9-ylamino)-5-amino-phenyl] methanol (**8a**) (1.130 g, 2.73 mmol)<sup>24</sup> in dry DMF (20 mL) containing pyridine (5 mL) at room temperature and stirred for 48 h. The reaction mixture was evaporated under reduced pressure to dryness and the solid residue was dissolved in a mixture of CHCl<sub>3</sub>/MeOH containing silica gel (5 g) and then evaporated in vacuo to dryness. The residue was put on the top of a silica gel column (20×4 cm) and purified by using CHCl<sub>3</sub>/MeOH (100/1 v/v) as an eluent. The fraction containing the main product were combined and evaporated in vacuo to dryness and the residue was recrystallized from CHCl<sub>3</sub>/MeOH to give **10a**, 1.169 g (79%); mp 241–242 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 3.72 (8H, s, 4 × CH<sub>2</sub>), 4.46 (2H, s, CH<sub>2</sub>), 5.36 (1H, br s, exchangeable, OH), 6.74 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.02 (1H, br s, ArH), 7.03 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.46–7.51 (4H, m, 4 × ArH), 7.99–8.02 (2H, m, 2 × ArH), 8.11–8.12 (2H, m, 2 × ArH), 8.28–8.30 (2H, m, 2 × ArH), 10.38 and 11.53 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>31</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

By following the same procedure, the following compounds were synthesized.

#### 5.1.2. [3-Hydroxymethyl-5-(4-methyl-acridin-9-ylamino)-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10b)

Compound **10b** was prepared from **9** (1.196 g, 3 mmol) and [3-amino-5-(4-methyl-acridin-9-ylamino)-phenyl]methanol (**8b**) (0.987 g, 3 mmol):<sup>24</sup> Yield 1.053 g (61%); mp 240–242 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.81 (3H, s, Me), 3.72 (8H, s, 4 × CH<sub>2</sub>), 4.57 (2H, s, CH<sub>2</sub>), 5.35 (1H, br s, exchangeable, OH), 6.74 (2H, d, *J* = 9.0 Hz, 2 × ArH), 6.98 (1H, br s, ArH), 7.03 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.38–7.40 (2H, m, 2 × ArH), 7.46–7.49 (2H, m, 2 × ArH), 7.86–7.87 (1H, m, ArH), 7.99–8.00 (1H, m, ArH), 8.21–8.27 (2H, m, 2 × ArH), 8.54–8.56 (1H, m, ArH), 10.37 and 11.77 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

#### 5.1.3. [2-Methyl-3-(4-methyl-acridin-9-ylamino)-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10c)

Compound **10c** was prepared from **9** (2.0 g, 5 mmol) and 2-methyl-*N*-(4-methyl-acridin-9-yl)-benzene-1,3-diamine (**8c**) (0.689 g, 2.2 mmol):<sup>25</sup> Yield 0.786 g (69%); mp 202–203 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.02 (3H, s, Me), 2.49 (3H, s, OMe), 3.73 (8H, s, 4 × CH<sub>2</sub>), 6.40–6.42 (1H, m, ArH), 6.74–6.77 (4H, m, 4 × ArH), 7.03 (2H, d, *J* = 8.6 Hz, 2 × ArH), 7.06–7.10 (3H, m, 3 × ArH), 7.33–7.45 (2H, m, 2 × ArH), 7.69–7.70 (1H, m, ArH), 8.06 (1H, br s, ArH), 9.33 and 9.71 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

#### 5.1.4. [5-(Acridin-9-ylamino)-2-methyl-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10d)

Compound **10d** was prepared from **9** (1.20 g, 3 mmol) and *N*<sup>1</sup>-acridin-9-yl-4-methyl-benzene-1,3-diamine (**8d**) (0.600 g, 2 mmol):<sup>25</sup> Yield 0.663 g (59%); mp 138–139 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.26 (3H, s, Me), 3.71 (8H, s, 4 × CH<sub>2</sub>), 6.47–6.50 (1H, m, ArH), 6.76–6.78 (3H, m, 3 × ArH), 6.94 (2H, br s, 2 × ArH), 7.00 (2H, d, *J* = 8.7 Hz, 2 × ArH), 7.11–7.13 (2H, m, 2 × ArH), 7.35–7.50 (4H, m, 4 × ArH), 8.21 (1H, m, ArH), 9.24 and 10.84 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>31</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

#### 5.1.5. [2-Methyl-5-(4-methyl-acridin-9-ylamino)-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10e)

Compound **10e** was prepared from **9** (1.197 g, 3 mmol) and 4-methyl-*N*<sup>1</sup>-(4-methyl-acridin-9-yl)-benzene-1,3-diamine (**8e**)

(0.940 g, 3 mmol):<sup>25</sup> Yield 0.905 g (52%); mp 154–156 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.36 (3H, s, Me), 2.81 (1H, s, Me), 3.71 (8H, s, 4 × CH<sub>2</sub>), 6.72 (2H, d, *J* = 9.0 Hz, 2 × ArH), 6.96–7.00 (2H, m, 2 × ArH), 7.12–7.13 (1H, m, 2 × ArH), 7.33–7.44 (3H, m, 3 × ArH), 7.62 (1H, br s, ArH), 7.83–7.84 (1H, m, ArH), 7.96–7.97 (1H, m, ArH), 8.21–8.27 (2H, m, 2 × ArH), 8.57–8.58 (1H, m, ArH), 9.52 and 11.65 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

#### 5.1.6. [4-Methyl-3-(4-methyl-acridin-9-ylamino)-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10f)

Compound **10f** was prepared from **9** (1.197 g, 3 mmol) and 4-methyl-*N*<sup>1</sup>-(4-methyl-acridin-9-yl)-benzene-1,3-diamine (**8f**) (0.627 g, 2 mmol):<sup>25</sup> Yield 0.563 g (49%); mp 104–105 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.21 (3H, s, Me), 2.80 (3H, s, Me), 3.70 (8H, s, 4 × CH<sub>2</sub>), 6.46–6.47 (1H, m, ArH), 6.70–6.72 (2H, m, 2 × ArH), 6.96 (5H, br s, 5 × ArH), 7.32–7.42 (2H, m, 2 × ArH), 7.60–7.72 (2H, m, 2 × ArH), 8.01–8.11 (2H, m, 2 × ArH), 9.04 and 9.15 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

#### 5.1.7. [5-(Acridin-9-ylamino)-2-methoxy-phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10g)

Compound **10g** was prepared from **9** (0.800 g, 2 mmol) and *N*<sup>1</sup>-acridin-9-yl-4-methoxy-benzene-1,3-diamine (8 g) (0.630 g, 2 mmol):<sup>26</sup> Yield 0.718 g (63%); mp 190–192 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 3.70 (8H, s, 4 × CH<sub>2</sub>), 3.92 (3H, s, OMe), 6.73 (2H, d, *J* = 8.3 Hz, 2 × ArH), 7.00 (2H, d, *J* = 8.7 Hz, 2 × ArH), 7.19 (2H, s, 2 × ArH), 7.39–7.44 (2H, m, 2 × ArH), 7.84 (1H, s, ArH), 7.94–7.98 (2H, m, 2 × ArH), 8.10–8.12 (2H, m, 2 × ArH), 8.26–8.29 (2H, m, 2 × ArH), 9.25 and 11.62 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>31</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

#### 5.1.8. [2-Methoxy-5-(4-methyl-acridin-9-ylamino)phenyl] carbamic acid 4-[bis(2-chloroethyl)amino] phenyl ester (10h)

Compound **10h** was prepared from **9** (1.197 g, 3 mmol) and 4-methoxy-*N*<sup>1</sup>-(4-methyl-acridin-9-yl)-benzene-1,3-diamine (8 h) (0.66 g, 2 mmol):<sup>26</sup> Yield 0.559 g (47%); mp 195–196 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.78 (3H, s, Me), 3.71 (8H, s, 4 × CH<sub>2</sub>), 3.79 (3H, s, OMe), 6.48 (1H, s, ArH), 6.70–6.72 (2H, m, 2 × ArH), 6.96–6.98 (3H, m, 3 × ArH), 7.28–7.39 (3H, m, 3 × ArH), 7.58–7.69 (2H, m, 2 × ArH), 8.04–8.13 (2H, m, 2 × ArH), 8.86 (1H, s, ArH), 8.99 and 9.67 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

#### 5.1.9. [3-(Acridin-9-ylamino)-5-methoxyphenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10i)

Compound **10i** was prepared from **9** (1.19 g, 3 mmol) and *N*<sup>1</sup>-acridin-9-yl-5-methoxy-benzene-1,3-diamine (**8i**) (0.945 g, 3 mmol):<sup>26</sup> Yield 0.317 g (21%); mp 179–180 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 3.66 (3H, s, OMe), 3.70–3.72 (8H, m, 4 × CH<sub>2</sub>), 6.13 (1H, br s, ArH), 6.57 (1H, br s, ArH), 6.74 (2H, d, *J* = 9.0 Hz, 2 × ArH), 6.90 (1H, br s, ArH), 7.02 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.10 (2H, br s, 2 × ArH), 7.51–7.59 (4H, m, 4 × ArH), 7.95 (2H, br s, 2 × ArH), 10.05 and 11.23 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>31</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

#### 5.1.10. [3-(Acridin-9-ylamino)-4-methoxyphenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10j)

Compound **10j** was prepared from **9** (2.0 g, 5 mmol) and *N*<sup>1</sup>-acridin-9-yl-4-methoxy-benzene-1,3-diamine (**8j**) (0.701 g, 2.2 mmol):<sup>26</sup> Yield 0.912 g (78%); mp 194–195 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 3.41 (3H, s, OMe), 3.73 (8H, s, 4 × CH<sub>2</sub>), 6.76 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.06 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.18–7.20 (1H, m, ArH), 7.43–7.47 (2H, m, 2 × ArH), 7.58–7.60 (1H, m, ArH), 7.76 (1H, s, ArH), 7.98–8.02 (2H, m, 2 × ArH), 8.11–8.13 (2H, m, 2 × ArH), 8.26–8.28 (2H, m, 2 × ArH), 10.31 and 11.37 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>31</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

### 5.1.11. [4-Methoxy-3-(4-methyl-acridin-9-ylamino) phenyl] carbamic acid 4-[bis(2-chloroethyl)-amino] phenyl ester (10k)

Compound **10k** was prepared from **9** (1.50 g, 3.7 mmol) and 4-methoxy-*N*<sup>1</sup>-(4-methyl-acridin-9-yl)-benzene-1,3-diamine (**8k**) (0.548 g, 1.6 mmol).<sup>26</sup> Yield 0.489 g (52%); mp 186–189 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.58 (3H, s, Me), 3.58 (3H, s, OMe), 3.71 (8H, s, 4 × CH<sub>2</sub>), 6.70–6.72 (4H, m, 4 × ArH), 6.95–6.97 (4H, m, 4 × ArH), 7.12 (2H, s, 2 × ArH), 7.44–7.56 (2H, m, 2 × ArH), 8.00–8.02 (2H, m, 2 × ArH), 8.22 and 9.80 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>32</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>3</sub>) C, H, N.

## 5.2. General procedure for the preparation of *N*-mustard linked to the hydroxyl function of AHMA-alkylcarbamates via a carbamic acid ester linkage (14a–c)

*N,N*-Bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**12**) was prepared by following the procedure developed by Jordan et al.<sup>16</sup> Isocyanate **13**<sup>30</sup> (freshly prepared from **12** by treating with triphosgene in CHCl<sub>3</sub> at –10 °C) was then reacted with appropriate 9-anilinoacridines carbamates (**11a–c**)<sup>27</sup> in dry DMF in the presence of triethylamine to give **14a–c**. The final products were purified either by recrystallization from an appropriate solvent or by column chromatography using (SiO<sub>2</sub>, CHCl<sub>3</sub>/MeOH, v/v 100:2).

The detailed procedure for the synthesis of **14a–c** is described as follows:

### 5.2.1. 4-[*N,N*-Bis(2-chloroethyl)amino]phenylisocyanate (13)

To a suspension of *N,N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**12**) (1.683 g, 5.4 mmol)<sup>16</sup> in dry chloroform (30 mL) was added triethylamine (2.5 mL) at 0 °C. The clear solution obtained was then added dropwise into a solution of triphosgene (0.623 g, 2.1 mmol) in dry chloroform (10 mL) at –10 °C. The reaction mixture was allowed to stand at room temperature. After being stirred for 30 min, the reaction mixture was evaporated to dryness under reduce pressure. The solid residue was triturated with dry THF (100 mL), filtered and washed with small amount of THF. The combined filtrate and washings was evaporated to dryness to give the crude isocyanate **13**<sup>30</sup> which was used directly for next reaction without further purification.

### 5.2.2. (3-(Acridin-9-ylamino)-5-{4-[bis(2-chloroethyl)amino]phenylcarbamoyl oxymethyl}-phenyl) carbamic acid ethyl ester (14a)

A solution of isocyanate **13** (freshly prepared from **12**, 0.44 g, 1.4 mmol) in dry DMF (5 mL) was added drop wise to a solution of [3-(acridin-9-ylamino)-5-hydroxymethyl-phenyl] carbamic acid ethyl ester (0.30 g, 0.8 mmol)<sup>27</sup> in dry DMF (15 mL) containing triethylamine (1 mL) at room temperature. The reaction mixture was stirred for 26 h at room temperature. The solid separated out was filtered and recrystallized from CHCl<sub>3</sub>/MeOH to give **14a**; 0.30 g (60%), mp 178–179 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 1.23 (3H, t, *J* = 7.0 Hz, Me), 3.69 (8H, s, 4 × CH<sub>2</sub>), 4.11 (2H, q, *J* = 7.0 Hz, CH<sub>2</sub>), 5.06 (2H, s, CH<sub>2</sub>), 6.69 (2H, d, *J* = 8.4 Hz, 2 × ArH), 7.04 (1H, s, ArH), 7.25 (2H, br s, 2 × ArH), 7.43–7.48 (2H, m, 2 × ArH), 7.54–7.57 (2H, m, 2 × ArH), 7.96–8.00 (2H, m, 2 × ArH), 8.14–8.16 (2H, m, 2 × ArH), 8.26–8.29 (2H, m, 2 × ArH), 9.40, 9.98 and 11.54 (each 1H, br s, exchangeable, 3 × NH). Anal. (C<sub>34</sub>H<sub>33</sub>Cl<sub>2</sub>N<sub>5</sub>O<sub>4</sub>) C, H, N.

By following the similar procedures, the following compounds were synthesized.

### 5.2.3. (3-(Acridin-9-ylamino)-5-{4-[bis(2-chloroethyl)amino]phenylcarbamoyloxymethyl}-phenyl)- carbamic acid iso-butyl ester (14b)

Compound **14b** was synthesized from **13** (freshly prepared from **12**, 1.530 g, 5 mmol) and [3-(acridin-9-ylamino)-5-hydroxymethyl-phenyl] carbamic acid iso-butyl ester (0.830 g, 2 mmol):<sup>27</sup>

Yield 0.158 g (13%), mp 120–122 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 0.89 (6H, d, *J* = 6.4 Hz, 2 × Me), 1.86–1.90 (1H, m, CH), 3.67 (8H, s, 4 × CH<sub>2</sub>), 3.82 (2H, d, *J* = 6.1 Hz, CH<sub>2</sub>), 5.01 (2H, s, CH<sub>2</sub>), 6.49 (1H, m, ArH), 6.67–6.69 (2H, m, 2 × ArH), 6.84 (2H, m, 2 × ArH), 7.18–7.26 (5H, m, 5 × ArH), 7.52 (3H, m, 3 × ArH), 8.30 (2H, m, 2 × ArH), 9.37, 9.57 and 10.94 (each 1H, br s, exchangeable, 3 × NH). Anal. (C<sub>36</sub>H<sub>37</sub>Cl<sub>2</sub>N<sub>5</sub>O<sub>4</sub>) C, H, N.

### 5.2.4. [3-{4-[Bis-(2-chloroethyl)amino]phenylcarbamoyloxymethyl}-5-(4-methylacridin-9-ylamino)phenyl] carbamic acid *tert*-butyl ester (14c)

Compound **14c** was synthesized from **13** (freshly prepared from **12**, 1.530 g, 5 mmol) and [3-hydroxymethyl-5-(4-methylacridin-9-ylamino)phenyl] carbamic acid *tert*-butyl ester (0.858 g, 2 mmol):<sup>27</sup> Yield 0.462 g (34%), mp 228–230 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 0.90 (6H, d, *J* = 6.7 Hz, 2 × Me), 1.82–1.89 (1H, m, CH), 2.81 (3H, s, Me), 3.68–3.70 (8H, m, 4 × CH<sub>2</sub>), 3.85 (2H, d, *J* = 6.6 Hz, CH<sub>2</sub>), 5.05 (2H, s, CH<sub>2</sub>), 6.69 (2H, d, *J* = 8.8 Hz, 2 × ArH), 6.99 (1H, s, ArH), 7.25 (2H, d, *J* = 6.1 Hz, 2 × ArH), 7.35–7.39 (1H, m, ArH), 7.44–7.54 (3H, m, 3 × ArH), 7.82 (1H, d, *J* = 7.0 Hz, ArH), 7.95–7.99 (1H, m, ArH), 8.19–8.25 (2H, m, 2 × ArH), 8.51–8.53 (1H, m, ArH), 9.38, 9.95 and 11.54 (each 1H, br s, exchangeable, 3 × NH). Anal. (C<sub>37</sub>H<sub>39</sub>Cl<sub>2</sub>N<sub>5</sub>O<sub>4</sub>) C, H, N.

### 5.2.5. Carbonic acid 3-(acridin-9-ylamino)-5-ethoxycarbonylamino-benzyl ester 4-[bis-(2-chloro-ethyl)-amino] phenyl ester (15a)

Compound **9** (0.80 g, 2 mmol) was added into a solution of [3-(acridin-9-ylamino)-5-hydroxymethylphenyl] carbamic acid ethyl ester (**11a**) (0.77 g, 2 mmol)<sup>27</sup> in dry DMF (20 mL) containing pyridine (5 mL) and DMAP (10 mg) at room temperature and stirred for 48 h. The Reaction mixture was evaporated under reduced pressure to dryness and the solid residue was dissolved in a mixture of CHCl<sub>3</sub>/MeOH containing silica gel (5 g) and then evaporated in vacuo to dryness. The residue was put on the top of a silica gel column (20 × 4 cm) and purified by using CHCl<sub>3</sub>/MeOH (100/1 v/v) as an eluent. The fraction containing the main product were combined and evaporated in vacuo to dryness and the residue was recrystallized from CHCl<sub>3</sub>/MeOH to give **15a**; 0.31 g (25%); mp 131–133 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 1.22 (3H, t, *J* = 7.0 Hz, Me), 3.71 (8H, s, 4 × CH<sub>2</sub>), 4.09 (2H, q, *J* = 7.0 Hz, CH<sub>2</sub>), 5.13 (2H, s, CH<sub>2</sub>), 6.53 (1H, br s, ArH), 6.73 (2H, d, *J* = 9.0 Hz, 2 × ArH), 6.96 (1H, br s, ArH), 7.01 (2H, d, *J* = 9.0 Hz, 2 × ArH), 7.04 (2H, br s, 2 × ArH), 7.25 (1H, s, ArH), 7.59 (4H, br s, 4 × ArH), 7.95 (2H, br s, 2 × ArH), 9.66 and 11.17 (each 1H, br s, exchangeable, 2 × NH). Anal. (C<sub>34</sub>H<sub>32</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>5</sub>) C, H, N.

## 6. Biological experiments

### 6.1. Cytotoxicity assays

The cytotoxic effects of the newly synthesized compounds were determined in T-cell acute lymphocytic leukemia (CCRF-CEM) and their resistant subcell lines (CCRF-CEM/Taxol and CCRF-CEM/VBL) by the XTT assay<sup>31</sup> and human solid tumor cells (i.e., breast carcinoma MX-1 and colon carcinoma HCT-116) the SRB assay<sup>32</sup> in a 72 h incubation using a microplate spectrophotometer as described previously.<sup>33</sup> After the addition of phenazine methosulfate-XTT solution, incubated at 37 °C for 6 h and absorbance at 450 and 630 nm was detected on a microplate reader (EL 340). The cytotoxicity of the newly synthesized compounds against non-small cell lung cancer (H1299), human prostate cancer cell line (PC3), human breast adenocarcinoma cell line (MCF-7), human lung adenocarcinoma (CL1-0 and CL1-5) cell lines were

determined by the WST-1 assay<sup>34</sup> in a 72 h incubation using a microplate spectrophotometer as described previously. After the addition of WST-1 solution, it was incubated at 37 °C for 4 h. Absorbance at 460 and 630 nm was detected on a microplate reader. IC<sub>50</sub> values were determined from dose-effect relationship at six or seven concentrations of each drug by using the COMPUSYN software by Chou and Martin<sup>35</sup> based on the median-effect principle and plot.<sup>36,37</sup> Ranges given for Taxol, Vinblastine and Cisplatin were mean ± SE (*n* = 4).

## 6.2. In vivo studies

Athymic nude mice bearing the nu/nu gene were used for human breast tumor MX-1 and human colon HCT-116 xenografts. Outbred Swiss-background mice were obtained from the National Cancer Institute (Frederick, MD). Male mice 8 weeks old or older weighing 22 g or more were used for most experiments. Drug was administered via the tail vein by iv injection. Tumor volumes were assessed by measuring length × width × height (or width) using caliper. Vehicle used was 50 μL DMSO and 40 μL Tween 80 in 160 μL saline. The maximal tolerate dose of the tested compound was determined and applied for the in vivo therapeutic efficacy assay. 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. Determination of DNA interstrand cross-linking

The level of DNA interstrand cross-linking was determined using a modified comet assay.<sup>38,39</sup> All steps were carried out under subdued lighting. Briefly, H1299 cells ( $2 \times 10^5$  cells) were plated in a 60 mm dish and incubated at 37 °C with 5% CO<sub>2</sub> for 32 h. The growing cells were treated with alkylating agent (**10a** or mephalan). After being treated with 1 h, the cells were exposed to 20 Gy X-ray to induce DNA strand breaks. An aliquot of  $5 \times 10^5$  cells were suspended in 50 μL of phosphate-buffered saline, mixed with a 250 μL of 1.2% low melting point agarose, and subjected to comet assay. The tail moment of 100 cells were analyzed for each treatment by aid of the COMET assay software. The degree of DNA interstrand cross-linking presented at a drug-treated sample was determined by comparing the tail moment of the irradiated control, which was calculated by the following formula. Percentage of DNA with interstrand cross-linking =  $[1 - (\text{TM}_{\text{di}} - \text{TM}_{\text{cu}}) / \text{TM}_{\text{ci}}] \times 100\%$ , where TM<sub>di</sub> = tail moment of drug-treated irradiated sample, TM<sub>cu</sub> = tail moment of untreated unirradiated control, and TM<sub>ci</sub> = tail moment of untreated irradiated control.

## 6.4. Determination of half-life of 10a in rat plasma

The chromatographic system consisted of a photodiode-array system, chromatographic pump, an auto-sampler equipped with a 40 μL sample loop. Compound **10a** was separated from rat plasma using a reverse phase column (30 × 150 mm, particle size 5 μm) maintained at an ambient temperature (25 ± 1 °C) to perform the ideal chromatographic system. The detector wavelength was set at 266 nm. The mobile phase comprised methanol:10 mM NaH<sub>2</sub>PO<sub>4</sub> (60:40, v/v), which was adjusted to pH 3.0 with 85% of H<sub>3</sub>PO<sub>4</sub>. Analysis was run at a flow rate of 0.5 mL/min and the samples were quantified using peak area. An aliquot of plasma sample (40 μL, with **10a** 5 μg/mL) was vortex-mixed with acetonitrile (1:2, v/v) for protein precipitation and centrifuged at 10,000g for 10 min. The supernatant was passed through a 0.45 μm filter for injected into the HPLC.

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## Supplementary data

Supplementary data (Elemental analysis data of compound **10a–k**, **14a–c** and **15a**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.022.

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