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Synthesis and biological activity of stable and potent antitumor agents, aniline nitrogen mustards linked to 9-anilinoacridines via a urea linkage

Naval Kapuriya,[a] Kalpana Kapuriya,[a] Xiuguo Zhang,[b] Ting-Chao Chou,[b] Rajesh Kakadiya,[a] Yu-Tse Wu,[c] Tung-Hu Tsai,[c] Yu-Ting Chen,[a] Te-Chang Lee,[a] Anamik Shah,[d] Yogesh Naliapara[d] and Tsann-Long Su[a,*]

[a]*Institute of Biomedical Sciences, Laboratory of Bioorganic Chemistry, Academia Sinica, Taipei 115, Taiwan*
[b]*Preclinical Pharmacology Core Laboratory, Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*
[c]*Institute of Traditional Medicine, National Yang-Ming University, Taipei 112, Taiwan*
[d]*Department of Chemistry, Saurashtra University, Rajkot, Gujarat, India*

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Abstract—To improve the chemical stability and therapeutic efficacy of N-mustard, a series of phenyl N-mustard linked to DNA-affinic 9-anilinoacridines and acridine via a urea linker were synthesized and evaluated for antitumor studies. The new N-mustard derivatives were prepared by the reaction of 4-bis(2-chloroethyl)aminophenyl isocyanate with a variety of 9-anilinoacridines or 9-aminoacridine. The antitumor studies revealed that these agents exhibited potent cytotoxicity in vitro without cross-resistance to taxol or vinblastine and showed potent antitumor therapeutic efficacy in nude mice against human tumor xenografts. It also showed that **24d** was capable of inducing marked dose-dependent levels of DNA cross-linking by comet assay and has long half-life in rat plasma.

1. Introduction

Bifunctional alkylating agents particularly N-mustards have played an important role in anticancer drug development.[1,2] However, they are highly reactive species resulting in loss of drug's therapeutic activity against malignancy by reacting with other cellular components such as proteins, thiols, or genes[3] and producing many unwanted side-effects including bone marrow toxicity.[4] In addition, the *N*,*N*-bis(2-chloroethyl)amine pharmacophore required for bifunctional cross-linking of DNA generally lacks intrinsic DNA-binding affinity. Consequently, DNA alkylation by N-mustards forms higher ratio for genotoxic mono-adducts than the bi-adducts, the latter was found to be essential for their full cytotoxicity.[5]

An effective strategy to overcome the drawback of using N-mustards is to design and synthesize DNA-directed alkylating agents by linking the N-mustard residue to DNA-affinic molecules. These conjugates were found to have higher cytotoxicity and therapeutic efficacy than the corresponding untargeted N-mustard derivatives.[6–10] Another strategy to minimize the side-effects caused by the drug is to prepare N-mustard prodrugs, which can be activated selectively at tumor site after enzymatic hydrolysis. Among N-mustard prodrugs, Springer et al. have synthesized a series of N-mustard prodrugs by attaching the aniline mustards to L-glutamic acid moiety through a urea, carbamate (**1**, Chart 1)[11] or carboxamide (**2**, CMDA)[12,13] linker for antibody-directed enzyme prodrug therapy (ADEPT). After enzymatic cleavage by bacterial enzyme carboxypeptidase G2 (CPG2), they can be transformed into their corresponding active metabolite phenol or aniline N-mustard drugs. The prodrugs, **3**,[14] **4**,[15] and **5**,[16] were also synthesized by linking the aniline N-mustard to the trigger unit tyramine, 3-hydroxytyramine, or catecholamine, respectively, via a

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* Corresponding author. Tel.: +886 2 27899045; fax: +886 2 27827685; e-mail: tlsu@ibms.sinica.edu.tw

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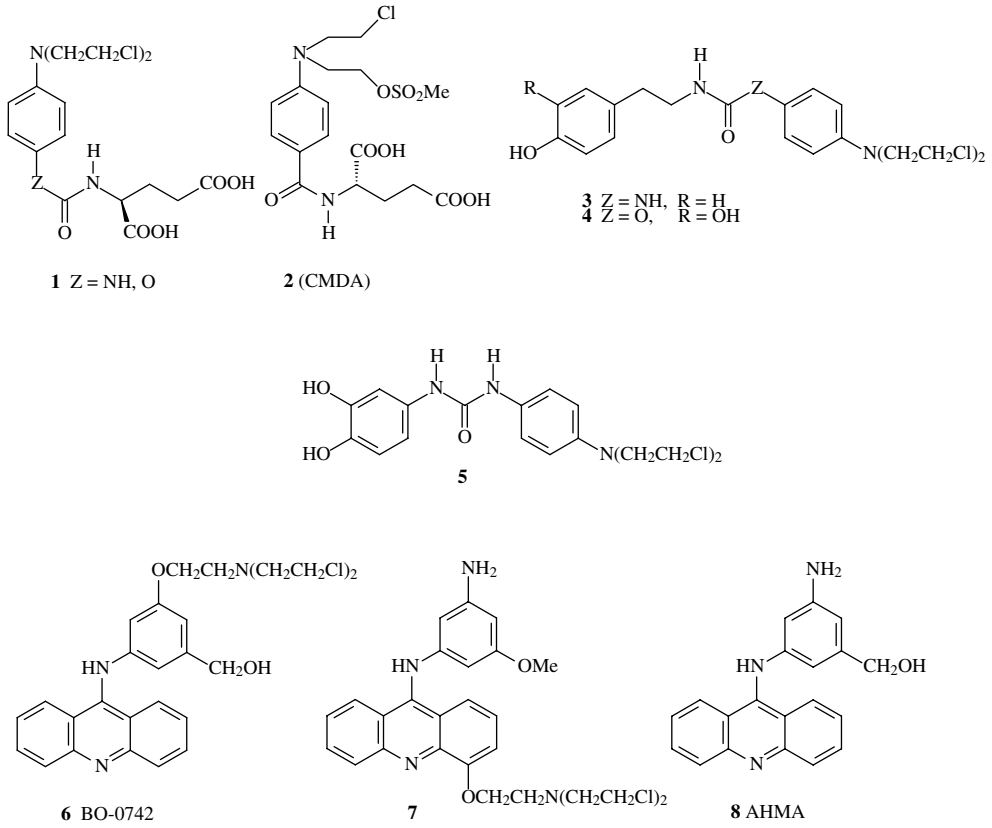


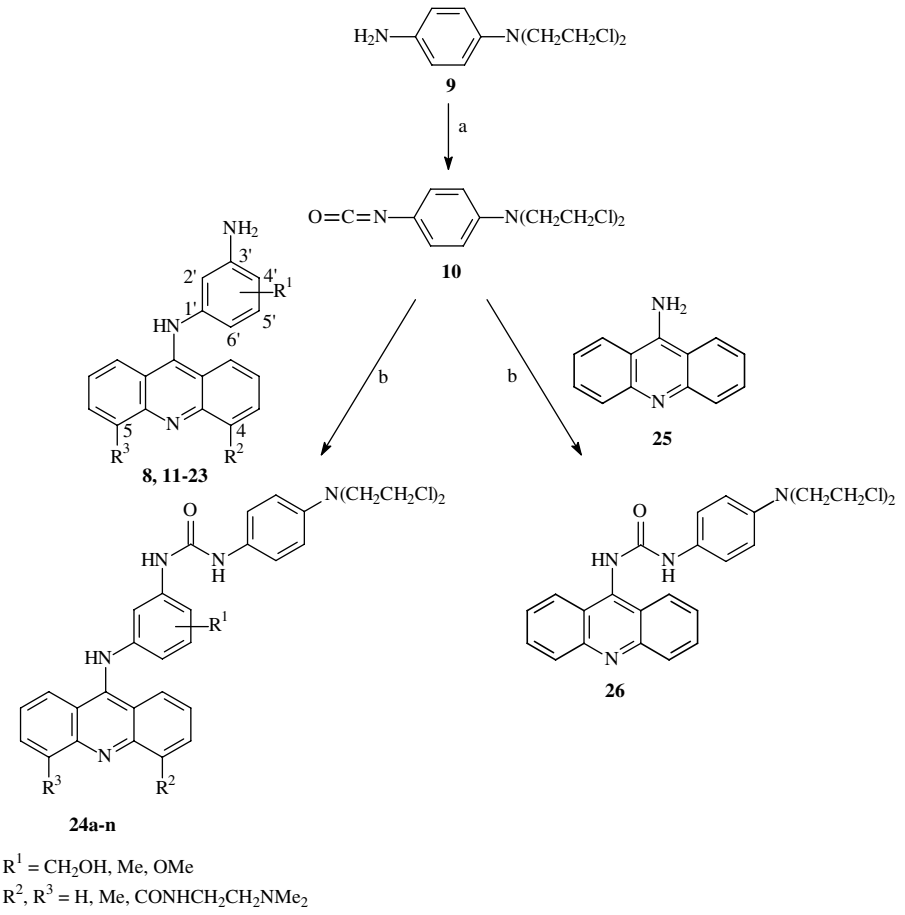
Chart 1. Chemical structures of N-mustard derivatives.

urea or carbamate linker for melanocyte-directed enzyme prodrug therapy (MDEPT).[16] Upon exposure to tyrosinase, these conjugates can also release the active aniline or phenol N-mustard. Since the prodrugs are stable before enzymatic hydrolysis, it suggests that the urea or carbamate linker is capable of lowering the reactivity of aniline or phenol N-mustard pharmacophore leading to form rather stable N-mustard derivatives.

Recently, we have reported a series of DNA-directed alkylating agents in which the alkyl N-mustard was linked to the anilino ring or acridine chromophore of 9-anilinoacridines, such as BO-0742 (**6**, Chart 1)[9] and BO-0944 (**7**).[10] These agents were about >100-fold more cytotoxic than AHMA (**8**)[17,18] in inhibiting human acute lymphoblastic leukemia (CCRF-CEM) in vitro. Remarkably, BO-0742 exhibited a broad spectrum of antitumor activity against various human solid tumor xenografts in vivo. Total tumor remission was achieved in nude mice bearing human breast MX-1 xenograft. Our unpublished results showed that BO-0742 is chemically unstable and has a short half-life (< 25 min.) in mice. The chemical instability of BO-0742 and the related compounds can be explained by the fact that these agents are considered as alkyl N-mustard derivatives. The inductive effect of the alkyl function is thought to be able to enhance the formation of the reactive aziridium cation intermediate, which reacts rapidly with nucleophile, such as the deoxyguanosine (dG) residue of DNA. While in the case of phenyl N-mustards, they are rather stable due to the electron-withdrawing property of phenyl ring. Therefore, in our continual development efforts on new potent DNA-targeted alkylating agents having an improved pharmacokinetics, it is of great interest to design and synthesize phenyl N-mustards linked to DNA-affinic molecule such as 9-anilinoacridines through a urea linker instead of alkyl N-mustard residue and carbon-chain spacer, since phenyl N-mustards having a urea linker at *para*-position are chemically less reactive than phenol or aniline N-mustards.[11,14,15] We found that the newly synthesized compounds were much more stable than BO-0742 and displayed potent therapeutic efficacy against several human xenografts in animal model. Herein, we describe the synthesis and antitumor activity of the new conjugates.

2. Chemistry

The newly synthesized compounds are phenyl N-mustards linked to 9-anilinoacridines via a urea spacer. In general, the urea linker can be prepared by reacting an amine derivative with either substituted carbamoyl chlorides or isocyanate derivatives (Scheme 1). We found that the desired compounds (**24a**–**n** and **26**) can be synthesized in better yields by treating 4-bis(2-chloroethyl)aminophenyl isocyanate **10** with a variety 9-anilinoacridines (**8**, **11**–**23**). Following the reported procedure with modification[14,15] aniline N-mustard **9** was synthesized, which was further converted into isocyanate **10**[21] by treating with triphosgene. The freshly prepared **10**



Scheme 1. Synthesis of N-mustard linked to 9-anilinoacridines and 9-aminoacridine via a urea linkage. Reagents and conditions: (a) triphosgene/$Et_3N/CHCl_3$, 0 °C; (b) Et_3N or pyridine/DMF, room temperature.

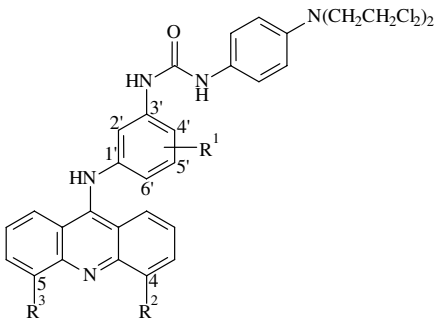
was then reacted with various 9-anilinoacridines (**8**, **11**–**23**), prepared by following the method previously developed in our laboratory,[17,19,20] in dry DMF in the presence of triethylamine or pyridine at room temperature to furnish the desired phenyl N-mustards linked to 9-anilinoacridine conjugates via a urea linker (**24a**–**n**). In a similar manner treatment of **10** with 9-aminoacridine (**25**) afforded acridine-N-mustard conjugate **26** in good yield. The yields and physical properties of the new N-mustard conjugates are shown in Table 1.

3. Biological results and discussion

3.1. In vitro cytotoxicity

We have previously demonstrated that antitumor 9-anilinoacridines including 3-(9-acridinylamino)-5-hydroxymethylanilines (AHMAs),[17] 5-(9-acridinylamino)toluidines,[19] and 5-(9-acridinylamino)anisidines[20] are potent inhibitors of topoisomerase II and capable of intercalating into DNA doubled strands. Hence, they are suitable as a carrier for constructing the new DNA-targeted compounds. Table 2 showed the cytotoxicity of the newly synthesized N-mustards (**24a**–**n** and **26**) against human lymphoblastic leukemia (CCRF-CEM), breast carcinoma (MX-1), and colon carcinoma HCT-116 and was compared with BO-0742 (**6**) and the untargeted N-mustard **9**. It was revealed that these conjugates possessed significant cytotoxicity with IC_{50} values in submicro molar range and did not exhibit cross-resistance to either vinblastine or taxol. The structure–activity relationships studies of the newly synthesized derivatives showed that the C4′-OMe and C5′-OMe substituted compounds were more cytotoxic than the corresponding C4′-Me and C5′-Me derivatives (**24h** vs **24c**, **24i** vs **24d**, **24j** vs **24e**) against CCRF-CEM cell growth in vitro. In contrast, the C4′-Me and C5′-Me substituted compounds were more potent than the corresponding C4′-OMe and C5′-OMe derivatives (**24c** vs **24h**, **24d** vs **24i**, **24e** vs **24j**) in inhibiting MX-1 cell growth. Although, the C6′-Me substituted compounds were more cytotoxic than the corresponding C6′-OMe derivatives (**24f** vs **24m** and **24g** vs **24n**) against CCRF-CEM cell growth, they (**24f** vs **24m**) were equally potent against MX-1 cell growth. The cytotoxicity of the series of OMe substituted compounds against CCRF-CEM showed that the C4′-OMe derivatives (**24h** and **24i**) were about 2- to 5-fold more potent than the corresponding C5′-OMe (**24j** and **24k**) and C6′-OMe (**24m** and **24n**) conjugates. As for the inhibitory effect of these conjugates against HCT-116 cell growth in culture, it showed that C4′-Me and C4′-OMe derivatives were more cytotoxic than the

Table 1. Analytical data and yields of N-mustard linked to 9-anilinoacridines (**24a**–**n** and **26**)



Compound	R^1	R^2	R^3	Mp (°C)	Yield (%)	Analysis
24a	5′-CH_2OH	H	H	174–175	53.8	C, H, N
24b	5′-CH_2OH	H	Me	251–252	55.2	C, H, N
24c	4′-Me	H	H	271–272	57.1	C, H, N
24d	4′-Me	H	Me	267–268	83.8	C, H, N
24e	5′-Me	H	H	>280	39.0	C, H, N
24f	6′-Me	H	Me	255–256	44.0	C, H, N
24g	6′-Me	Me	$CONH(CH_2)_2NMe_2$	>280	32.0	C, H, N
24h	4′-OMe	H	H	263–264	70.2	C, H, N
24i	4′-OMe	H	Me	255–256	60.2	C, H, N
24j	5′-OMe	H	H	260–261	64.5	C, H, N
24k	5′-OMe	H	Me	253–254	63.7	C, H, N
24l	5′-OMe	H	$CONH(CH_2)_2NMe_2$	188–190	61.8	C, H, N
24m	6′-OMe	H	H	268–269	80.4	C, H, N
24n	6′-OMe	H	Me	270–272	48.8	C, H, N
26				184–186	60	C, H, N

C5′-Me and C5′-OMe conjugates (**24c** vs **24e**, **24h** vs **24j** **24i** vs **24k**). It is of great interest to note that the cytotoxicity of these agents can be increased by addition of Me group at C4 (**24b**, **24f**, **24k**, and **24n**), or $CONHCH_2CH_2NMe_2$ and Me at C4 and C5 (**24h** and **24l**), respectively (except for compounds

Table 2. Cytotoxicity of new N-mustards 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[a]

Compound	IC_{50} (nM)				
	CCRF-CEM	CCRF-CEM/Taxol[b]	CCRF-CEM/VBL[b]	MX-1	HCT-116
24a	56.2 ± 1.1	17180 ± 142[304×] [c]	64140 ± 2000[1141×]	369.0 ± 11	216.0 ± 19
24b	31.1 ± 0.08	393.5 ± 6.6[12.7×]	1789 ± 331[57.5×]	124.7 ± 4.5	262.6 ± 6.0
24c	16.7 ± 0.60	34.7 ± 0.80[2.08×]	33.4 ± 1.0[2.00×]	583.6 ± 26.4	264.5 ± 4.7
24d	77.8 ± 2.3	91.8 ± 15.7[1.18×]	127.9 ± 2.2[1.64×]	239.7 ± 35.6	437.4 ± 1.8
24e	141.0 ± 3.3	384.2 ± 2.0[2.72×]	593.2 ± 2.0[4.20×]	529.0 ± 21.9	815.5 ± 10.1
24f	11.4 ± 0.46	23.0 ± 0.5[2.02×]	24.5 ± 1.1[2.15×]	588.6 ± 52.3	158.1 ± 3.2
24g	11.9 ± 0.30	175.7 ± 5.6[14.7×]	303.1 ± 0.30[25.4×]	60.1 ± 1.1	60.0 ± 2.8
24h	7.7 ± 0.14	33.8 ± 1.4[4.39×]	54.9 ± 1.8[7.13×]	1203 ± 40.6	256.3 ± 8.1
24i	9.2 ± 0.24	11.5 ± 0.50[1.25×]	24.6 ± 0.07[2.76×]	367.3 ± 12.5	74.9 ± 2.7
24j	37.5 ± 1.2	50.4 ± 0.80[1.34×]	54.8 ± 0.10[1.46×]	1109 ± 61.7	495.6 ± 0.53
24k	29.6 ± 0.60	49.8 ± 1.1[1.68×]	43.3 ± 0.50 [1.46×]	868.0 ± 47.8	242.0 ± 1.6
24l	13.2 ± 0.43	30.2 ± 0.20[2.29×]	951.4 ± 3.0[72.0×]	638.0 ± 2.0	110.2 ± 5.3
24m	44.2 ± 0.32	58.8 ± 0.20[1.33×]	54.8 ± 3.7[1.24×]	590.6 ± 4.2	187.8 ± 6.1
24n	29.7 ± 0.30	57.4 ± 0.10[1.93×]	30.6 ± 2.8[1.30×]	1810.4 ± 100	571.2 ± 18.5
26	228.9 ± 23.9	385.4 ± 21.3[1.68×]	378.1 ± 17.8[1.65×]	810.1 ± 19.3	899.8 ± 23.6
9	43.4 ± 0.50	33.8 ± 0.40[0.78×]	25.7 ± 0.10[0.59×]	84.4 ± 1.7	340.8 ± 4.9
6	3.33 ± 1.5	3.20 ± 0.40[0.96×]	12.8 ± 0.70[3.84×]	3.5 ± 0.60	6.5 ± 1.2
Taxol	1.3 ± 0.36	429.0 ± 112.6[330×]	1.274 ± 468[980×]	35.0 ± 0.51	1.3 ± 0.45
Vinblastine	0.73 ± 0.12	78.0 ± 14.8[106.2×]	496.0 ± 280.9[679.5×]	2.9 ± 0.083	1.8 ± 0.37

[a] Cell growth inhibition was measured by the XTT assay[22] for leukemic cells and the SRB assay[23] for solid tumor cells after 72-h incubation using a microplate spectrophotometer as described previously.[24] IC_{50} 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[25] based on the median-effect principle and plot[26,27] and serial deletion analysis. Ranges given for taxol and vinblastine were mean ± SE (n = 4).

[b] 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_{50} of the parent cell line.

[c] Numbers in the brackets are fold of cross-resistant determined by comparison with the corresponding IC_{50} of the parent cell line.

24a and **24d** against HCT-116 cell growth where the cytotoxicity of **24a** > **24b** and **24c** > **24d**). These results confirm our previous studies[17,19,20] and clearly demonstrated that the changes in the cytotoxicity profile by modifying substituent(s) in the anilino or acridine ring may attribute to the appropriate increasing lipophilicity and DNA-binding affinity. Of these derivatives, compound **24h** was the most cytotoxic with IC_{50} value of 7.7 nM. As for the in vitro cytotoxicity of N-mustard linked to acridine, it showed that compound **26** was less cytotoxic than compounds having 9-anilinoacridines as a carrier. It is worthwhile to note that most compounds linking to 9-anilinoacridines were equally potent or in some cases more cytotoxic than the unmasked N-mustard **9**, but this finding was not observed in compound **26**. This suggested that the 9-anilinoacridines were more favorable for using as a DNA-affinic carrier than acridine.

3.2. In vivo therapeutic activity

The therapeutic effects of the representative new N-mustards (**24a**, **24b**, **24d**, **24e**) against human breast carcinoma MX-1 and human Glioma U87 MG xenografts in nude mice were evaluated. Under the experimental conditions as indicated, **24b** and **24d** (Fig. 1) achieved complete tumor remission (CR) in nude mice bearing MX-1 xenograft at the doses of 30 and 100 mg/kg, intravenous injection (iv), Q2D × 5 (n = 3). Remarkably, both compounds (**24b** and **24d**) with only one cycle 5-dose-treatments, complete remission was achieved and maintained for over 70 days without any relapse in 3 out of 3 mice (Fig. 1A). Compound **24a** also led to complete tumor suppression but not complete tumor remission at the dose of 50 mg/kg, Q2D × 8 (n = 3). The maximal toxicity of these agents as shown in Figure 1B by body weight decrease was about 10% drop from the initial pretreatment body weight (on day 8), after four treatments. However, the body weight showed recovery after cession of treatment. Similar result was found for **24e**, which also resulted in tumor complete remission at the dose of 75 mg/kg (QD × 8, iv injection) in nude mouse bearing MX-1 xenograft (figure not shown). Compound **24d** was further selected to evaluate its therapeutic effect in nude mice bearing human glioma U87 MG xenograft (Fig. 2). The results showed that **24d** was more potent than cyclophosphamide with low toxicity to the host (15% body-weight drop). These studies

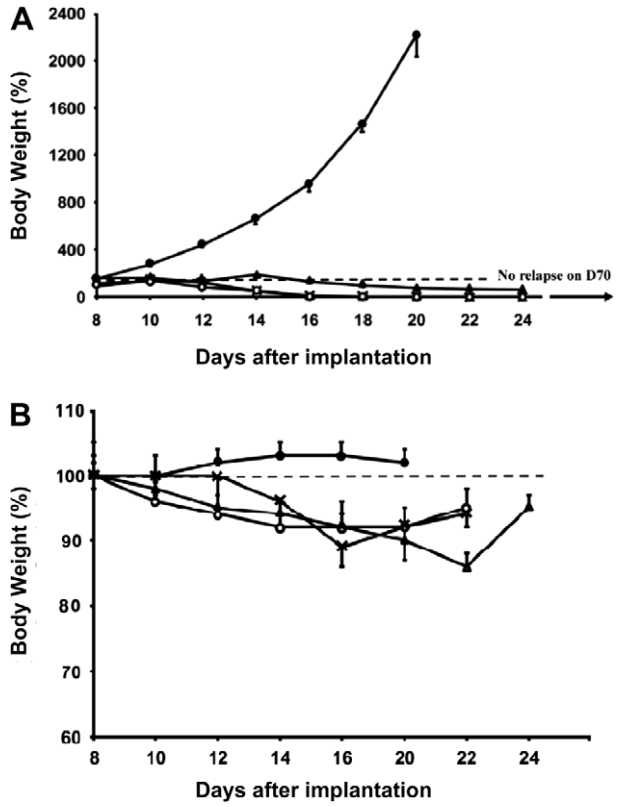


Figure 1. The therapeutic effects of **24a** (50 mg/kg, Q2D × 8, iv injection), **24b** (30 mg/kg, Q2D × 5, iv injection), and **24d** (100 mg/kg, Q2D × 5, iv injection) in nude mice bearing MX-1 xenograft (n = 3); control (●), **24a** (▲), **24b** (○), and **24d** (×); average tumor size changes (A); and average body weight changes (B). The values for the treated versus the untreated group from day 16 to day 24 are <0.0006, <0.0003 and <0.0003 for **24a**, **24b**, and **24d**, respectively.

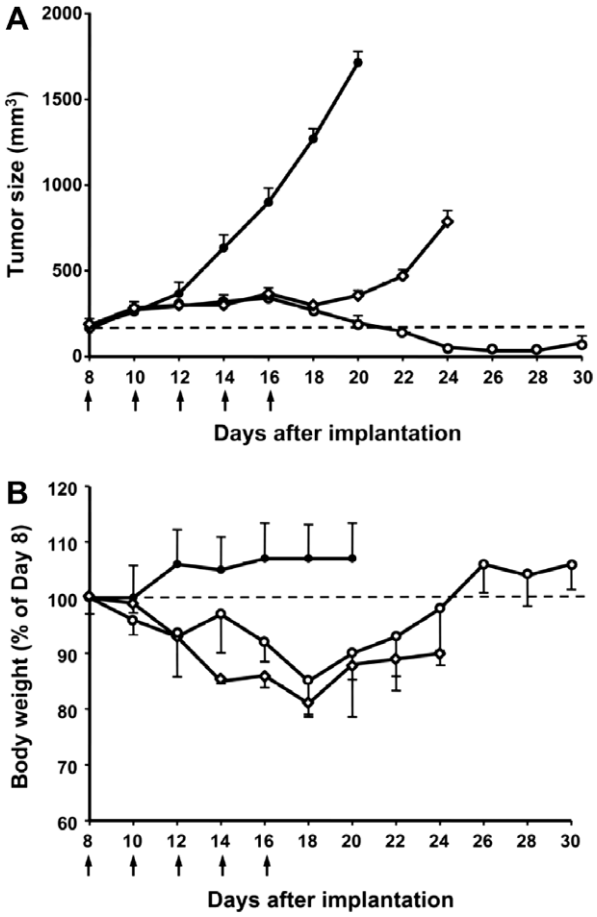


Figure 2. The therapeutic effects of **24d** (100 mg/kg) and cyclophosphamide (80 mg/kg) in nude mice bearing human glioma U87 MG xenograft (iv inj., Q2D × 5, n = 3); control (●), **24d** (○) and cyclophosphamide (◊); average tumor size changes (A); and body weight changes (B). The values for the treated versus the untreated group from day 18 to day 30 are <0.0001 and <0.0012 for cyclophosphamide and **24d**, respectively.

demonstrated that the newly synthesized compounds possess potent antitumor therapeutic efficacy with a relatively mild toxicity. Interestingly, we found that the complete tumor suppression was observed in mice on day-16 (last dose) and remained continuously for over 70 days without relapse.

3.3. Chemical stability

To realize whether the new conjugates are more stable than BO-0742, we further investigated the comparative chemical stability of the **24a**, **24b**, **24d**, and BO-0742 in intravenous injection vehicle (1 mg of compound in DMSO/Tween 80/normal saline: 0.5:0.4:1.6 v/v/v) by thin-layer chromatography (SiO_2, solvent: $CHCl_3$/MeOH, 10:1 v/v) and parallel confirmed by HPLC (Mightysil RP-18; mobile phase: acetonitril/H_2O 80:20, elution rate: 1 mL/min). It revealed that the half-life ($t_{1/2}$, time required for 50% decomposition of compound) of **24a**, **24b**, and **24d** were 55, 59, and 36 days, respectively. We could not detect the $t_{1/2}$ value for BO-0742 by HPLC analysis, since this agent decomposed during eluation from column. However, TLC analysis showed that BO-0742 had a $t_{1/2}$ value of 2 h demonstrating that the newly synthesized compounds were significantly more stable than BO-0742 although they were less cytotoxic than the latter.

Compound **24d** 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 for the authentic **24d** in the rat plasma. It revealed that **24d** is a very stable N-mustard derivative in rat plasma with a long half-life ($t_{1/2} = 54.18 \pm 0.96$ h, $n = 4$). These results demonstrate that the newly prepared N-mustards are chemically and metabolically stable derivatives.

3.4. DNA interstrand cross-linking study

Compound **24d** was found to be cytotoxic to human non-small lung cancer H1299 cell line with IC_{50} value of 0.51 μM. This agent was selected and subjected to DNA cross-linking studies in human non-small lung cancer H1299 cells by modified comet assay.[28] The DNA cross-linking caused by **24d** was compared with that of mephalan and cisplatin. It revealed that **24d** was capable of inducing DNA cross-linking in a dose-dependent manner (Fig. 3). At the dose of 10 μM, this agent induced 39.2% DNA cross-linking, while, mephalan and cisplatin induced 47.3% and 47.4% DNA cross-linking at the dose of 200 and 100 μM, respectively, under the same experimental conditions. The results suggested that DNA interstrand cross-linking may be the main mechanism of action of **24d** and the related compounds.

4. Conclusion

In this study, we have synthesized a series of chemically stable DNA-directed alkylating agents, in which the phenyl N-mustard residue is linked to DNA-intercalating 9-anilinoacridines via a urea spacer, demonstrating that these agents exhibited potent antitumor efficacy in vivo with a relatively low toxicity. Among these derivatives, compound **24d** was revealed to have potent antitumor effect in nude mice bearing human breast MX-1 xenograft; complete remission was achieved and maintained for over 70 days without any relapse with only one cycle of treatments. Compound **24d** also effectively suppressed human glioma U87 MG xenograft in nude mice. Moreover, we also found that this agent is able to cross-link with DNA and has a long half-life in rat plasma suggesting that this agent is a promising candidate for preclinical studies.

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, ASTM; Merck and 230–400 mesh, Silicycle Inc.). Thin-layer chromatography was performed on silica gel G60 F_{254} (Merck) with short-wavelength UV light for visualization. Elemental analyses were done

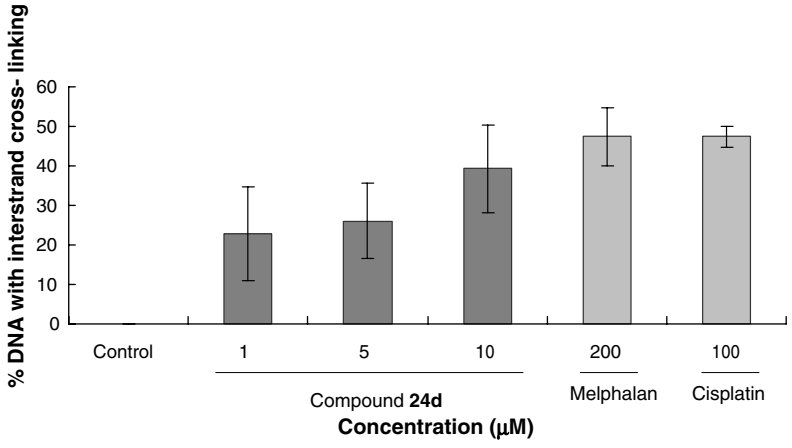


Figure 3. DNA interstrand cross-linking study. H1299 cells were used to determine the DNA cross-linking study by a modified comet assay. Mephalan and cisplatin were used as positive controls. Data represents the means of three individual experiments (mean ± SD).

on a Heraeus CHN-O Rapid instrument. HPLC was performed on Waters Delta Prep4000 using Mightysil RP-18 reverse phase column (250 × 4.6 mm). Compounds were detected by UV at 260 nm. The mobile phase was MeCN/H_2O (80:20 v/v) with flow rate of 1 mL/min. ^1H NMR spectra were recorded on a 600 MHz, Brucker AVANCE 600 DRX and 400 MHz, Brucker Top-Spin spectrometers. The chemical shifts were reported in ppm (δ) relative to TMS.

5.1. General procedure for the preparation of new N-mustards

N,*N*-Bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**9**) was prepared by following the procedure developed by Jordan et al.[14] Compound **9** was converted into isocyanate **10**, which was then condensed with appropriate 9-anilinoacridines (**8**, **11**–**23**) previously synthesized from our laboratory[17,19,20] and the commercially available 9-aminoacridine hydrochloride (**13**) in dry DMF in the presence of triethylamine or pyridine to give **24a**–**n** and **26**. The final products were purified either by recrystallization from an appropriate solvent or by column chromatography using (SiO_2, $CHCl_3$/MeOH, v/v 100:2). The detailed procedure was described as follows:

5.1.1. 4-[*N*,*N* -Bis(2-chloroethyl)amino]phenylisocyanate (10). To a suspension of *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**9**)[14] (1.683 g, 5.4 mmol) 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 reduced 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 **10**[21] which was used directly for the next reaction without further purification.

5.1.2. 1-[3-(Acridin-9-ylamino)-5-hydroxymethyl-phenyl]-3-{4-[bis(2-chloroethyl)amino]phenyl}urea (24a). A solution of isocyanate **10** (freshly prepared from **9**, 0.672 g, 2.2 mmol) in dry DMF (10 mL) was added dropwise to a solution of 3-(9-acridinylamino)-5-hydroxymethylaniline (**8**, 0.752 g, 2.0 mmol)[17] in dry DMF (40 mL) containing triethylamine (2.0 mL) at 0 °C. After being stirred for 18 h at room temperature, the reaction mixture was evaporated to dryness in vacuo, the residue was dissolved in a mixture of $CHCl_3$/MeOH containing silica gel (5.0 g) and then evaporated to dryness. The residue was put on the top of a silica gel column (2 × 20 cm) and purified by using $CHCl_3$/MeOH (100:5 v/v) as an eluent. The fractions containing the main product were combined and evaporated in vacuo to dryness and the residue was recrystallized from $CHCl_3$/MeOH to give **24a**, 618 mg (53.8%); mp 174–175 °C; ^1H NMR (DMSO-d_6) δ 3.65–3.71 (8H, m, 4× CH_2), 4.41 (2H, d, J = 6.0 Hz, CH_2), 5.16 (1H, t, J = 6.0 Hz, exchangeable, OH), 6.37 (1H, s, ArH), 6.68 (2H, d, J = 9.1 Hz, 2× ArH), 6.81 (1H, s, ArH), 7.01–7.05 (3H, m, 3× ArH), 7.24 (2H, d, J = 9.1 Hz, 2× ArH), 7.54 (4H, br s, 4× ArH) 8.05 (2H, br s, 2× ArH), 8.25, 8.46 and 10.84 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O_2{\cdot}0.5H_2O$) C, H, N.

By following the same procedure the following compounds were synthesized.

5.1.3. 1-{4-[Bis(2-chloroethyl)amino]phenyl}-3-[3-hydroxymethyl-5-(4-methylacridin-9-ylamino)phenyl]urea (24b). Compound **24b** was synthesized from **10** (freshly prepared from **9**, 1.377 g, 4.5 mmol) and 5-hydroxymethyl 3-(4-methyl-9-acridinylamino)aniline (**11**, 0.988 g, 3.0 mmol)[17] in dry DMF (25 mL) containing pyridine (2.0 mL): yield 986 mg (55.2%); mp 251–252 °C; ^1H NMR (DMSO-d_6) δ 2.79 (3H, s, Me), 3.66–3.69 (8H, m, 4× CH_2), 4.45 (2H, d, J = 5.9 Hz, CH_2), 5.30 (1H, br s, exchangeable, OH), 6.68 (2H, d, J = 8.8 Hz, 2× ArH), 6.87 (1H, s, ArH), 7.24 (2H, d, J = 8.8 Hz, 2× ArH), 7.34 (1H, s, ArH), 7.39–7.41 (1H, m, ArH), 7.47–7.48 (1H, m, ArH), 7.57 (1H, s, ArH), 7.86 (1H, d, J = 7.0 Hz, ArH), 8.00 (1H, s, ArH), 8.21–8.26 (2H, m, 2× ArH), 8.45 (1H, d, J = 8.5 Hz, ArH), 8.82, 9.62 and 11.49 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{32}H_{31}Cl_2N_5O_2{\cdot}3H_2O$) C, H, N.

5.1.4 1-(5-(Acridin-9-ylamino)-2-methylphenyl)-3-(4-(bis-(2-chloroethyl)amino)phenyl)urea (24c). Compound **24c** was synthesized from **10** (freshly prepared from **9**, 1.683 g, 5.4 mmol) and N^1-(acridin-9-yl)amino-4-methylbenzene-1,3-diamine (**12**, 0.898 g 3.0 mmol)[19] in dry DMF (15 mL) containing triethylamine (2.5 mL): yield 771 mg (57.1%); mp 271–272 °C (dec); ^1H NMR (DMSO-d_6) δ 2.35 (3H, s, Me), 3.66–3.69 (8H, m, 4× CH_2), 6.69 (2H, d, J = 8.8 Hz, 2× ArH), 6.86–6.88 (1H, m, ArH), 7.25–7.27 (1H, m, ArH), 7.28 (2H, d, J = 8.8 Hz, 2× ArH), 7.42–7.45 (2H, m, 2× ArH), 7.90–8.00 (4H, m, 4× ArH), 8.19 (1H, s, ArH), 8.26 (2H, m, 2× ArH), 8.30, 9.37 and 11.49 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O{\cdot}2.5H_2O$) C, H, N.

5.1.5. 1-{4-[Bis(2-chloroethyl)amino]phenyl}-3-[2-methyl-5-(4-methylacridin-9-ylamino)phenyl]urea (24d). Compound **24d** was synthesized from **10** (freshly prepared from **9**, 1.836 g, 6 mmol) and 4-methyl-N^1-(4-methylacridin-9-yl)benzene-1,3-diamine (**13**, 1.065 g, 3.4 mmol)[19] in dry DMF (50 mL) containing pyridine (2.0 mL): yield 1.631 g (83.8%); mp 267–268 °C; ^1H NMR (DMSO-d_6) δ 2.34 (3H, s, Me), 2.78 (3H, s, Me), 3.65–3.70 (8H, m, 4× CH_2), 6.68 (2H, d, J = 9.2 Hz, 2× ArH), 6.86–6.89 (1H, m, ArH), 7.24–7.27 (1H, m, ArH), 7.28 (2H, d, J = 9.2 Hz, 2× ArH), 7.37–7.41 (1H, m, ArH), 7.43–7.48 (1H, m, ArH), 7.86–7.87 (1H, m, ArH), 7.97–8.01 (1H, m, ArH), 8.20–8.23 (3H, m, 3× ArH), 8.33 (1H, br s, exchangeable, NH), 8.42–8.44 (1H, m, ArH), 9.35 and 11.51 (each 1H, br s, exchangeable, 2× NH). Anal. ($C_{32}H_{31}Cl_2N_5O{\cdot}2H_2O$) C, H, N.

5.1.6 1-[3-(Acridin-9-yl)amino-5-methylphenyl]-3-{4-[bis(2-chloroethyl)amino]phenyl}urea (24e). Compound **24e** was synthesized from **10** (freshly prepared from **9**, 0.918 g, 3.0 mmol) and N^1-(acridin-9-yl)amino-

5-methylbenzene-1,3-diamine (**14**, 0.517 g, 1.7 mmol)[19] in dry DMF (25 mL) containing pyridine (2 mL): yield 375 mg (39%); mp > 280 °C; ^{1}H NMR (DMSO-d_6) δ 2.26 (3H, s, Me), 3.68 (8H, s, 4× CH $_2$), 6.68 (2H, d, J = 9.0 Hz, 2× ArH), 6.79 (1H, s, ArH), 7.25–7.27 (3H, m, 3× ArH), 7.46–7.49 (2H, m, 2× ArH), 7.54 (1H, s, ArH), 8.00–8.07 (4H, m, 4× ArH), 8.28–8.30 (2H, m, 2× ArH), 8.95, 9.38 and 11.51 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O$·2.9-H_2O) C, H, N.

5.1.7. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(4-methyl-3-(4-methylacridin-9-ylamino)phenyl)urea (24f). Compound **24f** was synthesized from **10** (freshly prepared from **9**, 1.683 g, 5.4 mmol) and 6-methyl-N^1-(4-methylacridin-9-yl)benzene-1,3-diamine (**15**, 0.910 g 3.0 mmol)[19] in dry DMF (15 mL) containing triethylamine (2.5 mL): yield 576 mg (44%); mp 255–256 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.34 (3H, s, Me), 2.78 (3H, s, Me), 3.65–3.71 (8H, m, 4×CH_2), 6.69 (2H, d, J = 9.0 Hz, 2× ArH), 6.81–6.83 (1H, m, ArH), 7.21–7.24 (1H, m, ArH), 7.28 (2H, d, J = 9.0 Hz, 2× ArH), 7.34–7.38 (1H, m, ArH), 7.41–7.45 (1H, m, ArH), 7.82–7.83 (1H, m, ArH), 7.93–7.97 (1H, m, ArH), 8.15–8.22 (3H, m, 3× ArH), 8.31 (1H, br s, exchangeable, NH), 8.39–8.42 (1H, m, ArH), 9.40 and 12.45 (each 1H, br s, exchangeable, 2× NH). Anal. ($C_{32}H_{31}Cl_2N_5O$·2.7H_2O) C, H, N.

5.1.8. 9-[5-(3-{4-[Bis(2-chloroethyl)amino]phenyl}ureido)-2-methylphenylamino]-5-methylacridine-4-carboxylic acid (2-dimethylaminoethyl)amide (24g). Compound **24g** was synthesized from **10** (freshly prepared from **9**, 0.918 g, 3.0 mmol) and 9-(5-amino-2-methylphenylamino)-5-methylacridine-4-carboxylic acid (2-dimethylaminoethyl)amide (**16**, 0.732 g, 1.7 mmol)[19] in dry DMF (25 mL) containing pyridine (2 mL): yield 385 mg (32%); mp > 280 °C; ^{1}H NMR (DMSO-d_6) δ 2.19 (3H, s, Me), 2.67 (6H, br s, 2× NMe), 2.85 (3H, s, Me), 3.14 (2H, br s, CH_2), 3.63–3.70 (8H, m, 4× CH_2), 3.85 (2H, s, CH_2), 6.40–6.41 (1H, m, ArH), 6.65 (2H, d, J = 9.0 Hz, 2× ArH), 6.97–6.99 (1H, s, ArH), 7.24 (2H, d, J = 9.0 Hz, 2× ArH), 7.45 (1H, s, ArH), 7.51 (1H, m, ArH), 7.74 (2H, br s, 2× ArH), 7.93 (1H, br s, exchangeable, NH), 8.08–8.10 (1H, m, ArH), 8.39–8.41 (1H, m, ArH), 8.67–8.69 (1H, m, ArH), 9.04, 9.45 and 12.18 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{37}H_{41}Cl_2N_7O_2$·5.7H_2O) C, H, N.

5.1.9. 1-(5-(Acridin-9-ylamino)-2-methoxyphenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl)urea (24h). Compound **24h** was synthesized from **10** (freshly prepared from **9**, 1.683 g, 5.4 mmol) N^1-(acridin-9-yl)-4-methoxybenzene-1,3-diamine (**17**, 0.946 g, 3.0 mmol),[20] in dry DMF (15 mL) containing triethylamine (2.5 mL): yield 923 mg (70.2%); mp 263–264 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.67–3.68 (8H, m, 4× CH_2), 3.96 (3H, s, OMe), 6.68 (2H, d, J = 8.5 Hz, 2× ArH), 6.95–6.96 (1H, m, ArH), 7.12–7.13 (1H, m, ArH), 7.26 (2H, d, J = 8.5 Hz, 2× ArH), 7.42–7.44 (2H, m, 2× ArH), 7.95–7.97 (2H, m, 2× ArH), 8.00–8.02 (2H, m, 2× ArH), 8.24–8.27 (2H, m, 2× ArH), 8.33 (1H, br s, ArH), 8.44, 9.29 and 11.47 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O_2$·2.6H_2O) C, H, N.

5.1.10. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(2-methoxy-5-(4-methylacridin-9-ylamino)phenyl)urea (24i). Compound **24i** was synthesized from **10** (freshly prepared from **9**, 0.841 g, 2.7 mmol) and 4-methoxy-N^1-(4-methylacridin-9-yl)benzene-1,3-diamine (**18**, 0.493 g 1.5 mmol)[20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 428 mg (60.2%); mp 255–256 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.50 (3H, s, Me), 3.65–3.68 (8H, m, 4× CH_2), 3.94 (3H, s, OMe), 6.68 (2H, d, J = 9.0 Hz, 2× ArH), 6.93–6.95 (1H, m, ArH), 7.10 (1H, m, ArH), 7.26 (2H, d, J = 9.0 Hz, 2× ArH), 7.35–7.37 (1H, m, ArH), 7.41–7.43 (1H, m, ArH), 7.82–7.83 (1H, s, ArH), 7.94–7.96 (1H, m, ArH), 8.21–8.22 (2H, m, 2× ArH), 8.31–8.32 (1H, m, ArH), 8.50 (1H, br s, ArH), 8.51, 9.44 and 11.55 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{32}H_{31}Cl_2N_5O_2$·2.4H_2O) C, H, N.

5.1.11. 1-(3-(Acridin-9-ylamino)-5-methoxyphenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl)urea (24j). Compound **24j** was synthesized from **10** (freshly prepared from **9**, 0.841 g, 2.7 mmol) and N^1-(acridin-9-yl)-5-methoxybenzene-1,3-diamine (**19** , 0.473 g 1.5 mmol)[20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 452 mg (64.5%); mp 260–261 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.67 (3H, s, OMe), 3.68–3.70 (8H, m, 4× CH_2), 6.56 (1H, s, ArH), 6.69 (2H, d, J = 8.9 Hz, 2× ArH), 7.15 (1H, s, ArH), 7.19 (1H, s, ArH), 7.25 (2H, d, J = 8.9 Hz, 2× ArH), 7.47–7.50 (2H, m, 2× ArH), 7.98–8.00 (2H, m, 2× ArH), 8.04–8.06 (2H, m, 2× ArH), 8.29–8.31 (2H, m, 2× ArH), 8.88, 9.39 and 11.45 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O_2$·2.2H_2O): C, H, N.

5.1.12. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-methoxy-5-(4-methylacridin-9-ylamino)phenyl)urea (24k). Compound **24k** was synthesized from **10** (freshly prepared from **9**, 0.841 g, 2.7 mmol) and 5-methoxy-N^1-(4-methylacridin-9-yl)benzene-1,3-diamine (**20**, 0.493 g 1.5 mmol), [20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 459 mg (63.7%); mp 253–254 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.80 (3H, s, Me), 3.66 (3H, s, OMe), 3.67–3.70 (8H, m, 4× CH_2), 6.54 (1H, s, ArH), 6.69 (2H, d, J = 9.0 Hz, 2× ArH), 7.14 (1H, s, ArH), 7.17 (1H, s, ArH), 7.25 (2H, d, J = 9.0 Hz, 2× ArH), 7.40–7.42 (1H, m, ArH), 7.47–7.50 (1H, m, ArH), 7.85–7.86 (1H, m, ArH), 7.98–8.00 (1H, m, ArH), 8.23–8.27 (2H, m, 2× ArH), 8.48–8.50 (1H, m, ArH), 8.97, 9.51 and 11.47 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{32}H_{31}Cl_2N_5O_2$·2.5H_2O) C, H, N.

5.1.13. 1-(3-(4-((2-(Dimethylamino)ethyl)carbamoyl)acridin-9-ylamino)-5-methoxyphenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl)urea (24l). Compound **24l** was synthesized from **10** (freshly prepared from **9**, 0.550 g, 1.8 mmol) and 9-(3-amino-5-methoxyphenylamino)-N^1-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**21**, 0.429 g 1.0 mmol)[20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 359 mg (61.8%); mp 188–190 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.88 (6H, br s, 2× NMe), 3.44 (2H, s, CH_2), 3.60–3.68 (11H, m, 4× CH_2, OMe), 3.73 (1H, br s, CH), 3.97 (1H, s, CH), 6.04 (1H, s, exchangeable, NH), 6.67–6.68 (2H, m, 2×

ArH), 6.78–6.87 (1H, m, ArH), 7.21 (2H, br s, 2× ArH), 7.53–7.57 (2H, m, 2× ArH), 7.87 (1H, s, ArH), 8.24 (1H, s, ArH), 8.34–8.43 (1H, m, ArH), 8.68 (1H, s, ArH), 8.86 (1H, br s, ArH), 9.11–9.33 (1H, m, ArH), 9.57 (1H, s, ArH), 10.48 and 12.01 (each 1H, br s, exchangeable, 2× NH). Anal. ($C_{36}H_{39}Cl_2N_7O_3 \cdot 6.9H_2O$) C, H, N.

5.1.14. 1-(3-(Acridin-9-ylamino)-4-methoxyphenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl)urea (24m). Compound **24m** was synthesized from **10** (freshly prepared from **9**, 0.841 g, 2.7 mmol) and N^1-(acridin-9-yl)-6-methoxybenzene-1,3-diamine (**22**, 0.473 g 1.5 mmol)[20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 563 mg (80.4%); mp 268–269 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.41 (3H, s, OMe), 3.67–3.70 (8H, m, 4× CH_2), 6.70 (2H, d, J = 9.0 Hz, 2× ArH), 7.14–7.15 (1H, m, ArH), 7.28 (2H, d, J = 9.0 Hz, 2× ArH), 7.43–7.47 (3H, m, 3× ArH), 7.81 (1H, br s, ArH), 7.98–8.00 (2H, m, 2× ArH), 8.04–8.06 (2H, m, 2× ArH), 8.26–8.28 (2H, m, 2× ArH), 8.88, 9.30 and 11.32 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{31}H_{29}Cl_2N_5O_2 \cdot 3.4H_2O$) C, H, N.

5.1.15. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(4-methoxy-3-(4-methylacridin-9-ylamino)phenyl)urea (24n). Compound **24n** was synthesized from **10** (freshly prepared from **9**, 0.841 g, 2.7 mmol) and 6-methoxy-N^1-(4-methylacridin-9-yl)benzene-1,3-diamine (**23**, 0.493 g 1.5 mmol)[20] in dry DMF (10 mL) containing triethylamine (2.0 mL): yield 347 mg (48.8%); mp 270–272 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.79 (3H, s, Me), 3.39 (3H, s, OMe), 3.67–3.70 (8H, m, 4× CH_2), 6.69 (2H, d, J = 8.8 Hz, 2× ArH), 7.12–7.13 (1H, m, ArH), 7.28 (2H, d, J = 8.8 Hz, 2× ArH), 7.37–7.39 (1H, m, ArH), 7.42–7.45 (2H, m, 2× ArH), 7.78 (1H, br s, ArH), 7.85–7.86 (1H, m, ArH), 7.97–7.99 (1H, m, ArH), 8.18–8.23 (2H, m, 2× ArH), 8.46–8.50 (1H, m, ArH), 8.93, 9.35 and 11.32 (each 1H, br s, exchangeable, 3× NH). Anal. ($C_{32}H_{31}Cl_2N_5O_2 \cdot 3.2H_2O$) C, H, N.

5.1.16. 1-Acridin-9-yl-3-{4-[bis-(2-chloroethyl)amino]-phenyl}-urea (26). Compound **26** was synthesized from **10** (freshly prepared from **9**, 0.306 g, 1 mmol) and 9-aminoacridinehydrochloride (**25**, 0.248 g, 1 mmol) in dry DMF (10 mL) containing triethylamine (0.5 mL): yield 273 mg (60%); mp 184–186 °C; ^{1}H NMR (DMSO-d_6) δ 3.69–3.71 (8H, m, 4× CH_2), 6.72 (2H, d, J = 8.8 Hz, 2× ArH), 7.10–7.14 (2H, m, 2× ArH), 7.39–7.48 (3H, m, 3× ArH), 7.58–7.62 (2H, m, 2× ArH), 7.84–7.86 (1H, m, ArH), 8.14–8.16 (2H, m, 2× ArH), 9.37 and 11.33 (each 1H, br s, exchangeable, 2× NH). Anal. ($C_{24}H_{22}Cl_2N_4O$) C, H, N.

5.2. Biological experiments

5.2.1. Cytotoxicity assays. The effects of the compounds on cell growth 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[22] and human solid tumor cells (i.e., breast carcinoma MX-1 and colon carcinoma HCT-116) the SRB assay[23] in a 72 h incubation using a microplate spectrophotometer as described previously.[24] 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). IC_{50} values were determined from dose–effect relationship at six or seven concentrations of each drug by using the CompuSyn software by Chou and Martin[25] based on the median-effect principle and plot.[26,27] Ranges given for taxol and vinblastine were mean ± SE (n = 4).

5.2.2. In vivo studies. Athymic nude mice bearing the nu/nu gene were used for human breast tumor MX-1 and human glioma U87 MG xenograft. 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 administrated via the tail vein by iv injection. Tumor volumes were assessed by measuring length × width × height (or width) by 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.

5.2.3. Determination of half-life of 24d in rat plasma. The chromatographic system consisted of a photodiode-array system (Shimadzu SPD-M20A, Tokyo, Japan), chromatographic pump (Shimadzu LC-20AT), an auto-sampler (Shimadzu SIL-20AC) equipped with a 20 μL sample loop. Compound **24d** was separated from rat plasma using a revise phase column (Agilent RP-C8, 30 × 150 mm, particle size 5 μm, Palo Alto, CA, USA) 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_2PO_4 (60:40, v/v), which was adjusted to pH 3.0 with 85% of H_3PO_4. Analysis was run at a flow rate of 0.8 mL/min and the samples were quantified using peak area. An aliquot of plasma sample (100 μL, with **24d** 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 injecting into the HPLC.

5.3. Determination of DNA interstrand cross-linking

The level of DNA interstrand cross-linking was determined using a modified comet assay.[28,29] All steps were carried out under subdued lighting. Briefly, H1299 cells (2×10^5 cells) were plated in a 60 mm dish and incubated at 37 °C with 5% CO_2 for 32 h. The growing cells were treated with alkylating agent (**24d**, mephalan or cisplatin). After being treated for 1 h, the cells were exposed to 20 Gy irradiation to induce DNA strand breaks. An aliquot of 5×10^5 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 (Perceptive instruments, Haverhill, UK). 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 − (TMdi − TMcu/TMci − TMcu)]×100%, where TMdi = tail moment of drug-treated irradiated sample, TMcu = tail moment of untreated unirradiated control, and TMci = tail moment of untreated irradiated control.

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Appendix A

C, H, N analysis

Compound	Molecular formula	MW	C, H, N analysis					
			Anal. Calcd			Found		
			C%	H%	N%	C%	H%	N%
24a	$C_{31}H_{29}Cl_2N_5O_2{\cdot}0.5H_2O$	583.53	63.81	5.18	12.00	64.07	5.26	11.67
24b	$C_{32}H_{31}Cl_2N_5O_2{\cdot}3H_2O$	642.59	59.81	5.80	10.89	59.74	5.79	10.67
24c	$C_{31}H_{29}Cl_2N_5O{\cdot}2.5H_2O$	603.54	61.69	5.20	11.60	61.83	5.17	11.53
24d	$C_{32}H_{31}Cl_2N_5O{\cdot}2H_2O$	608.57	63.15	3.42	11.00	63.38	3.52	10.97
24e	$C_{31}H_{29}Cl_2N_5O{\cdot}2.9H_2O$	609.78	61.06	5.23	11.48	61.08	5.11	11.32
24f	$C_{32}H_{31}Cl_2N_5O{\cdot}2.7H_2O$	621.18	61.87	5.43	11.27	61.84	5.44	11.43
24g	$C_{37}H_{41}Cl_2N_7O_2{\cdot}5.7H_2O$	789.38	56.21	6.69	11.42	56.25	6.72	11.44
24h	$C_{31}H_{29}Cl_2N_5O_2{\cdot}2.6H_2O$	601.36	61.92	5.23	11.64	61.96	5.21	11.59
24i	$C_{32}H_{31}Cl_2N_5O_2{\cdot}2.4H_2O$	631.75	60.83	5.24	11.08	60.89	5.25	10.93
24j	$C_{31}H_{29}Cl_2N_5O_2{\cdot}2.2H_2O$	614.15	60.62	5.05	11.40	60.64	5.05	11.24
24k	$C_{32}H_{31}Cl_2N_5O_2{\cdot}2.5H_2O$	633.56	60.66	5.22	11.05	60.64	5.23	10.95
24l	$C_{36}H_{39}Cl_2N_7O_3{\cdot}6.9H_2O$	812.96	53.19	5.57	12.06	53.18	5.56	12.03
24m	$C_{31}H_{29}Cl_2N_5O_2{\cdot}3.4H_2O$	635.77	58.56	5.22	11.01	58.50	5.24	11.09
24n	$C_{32}H_{31}Cl_2N_5O_2{\cdot}3.2H_2O$	646.17	59.48	5.32	10.84	59.49	5.35	10.82
26	$C_{24}H_{22}Cl_2N_4O$	453.38	63.58	4.89	12.36	63.35	5.05	12.09

References and notes

1. Gajski, S. R.; William, R. M. *Chem. Rev.* **1998**, *98*, 2723.
2. Denny, W. A. *Curr. Med. Chem.* **2001**, *8*, 533.
3. Suzukake, K.; Vistica, B. P.; Vistica, D. T. *Biochem. Pharmacol.* **1983**, *32*, 165.
4. Rodney, M.; Carney, J. P.; Kelley, M. R.; Glassner, B. J.; Williams, D. A.; Samson, L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 206.
5. Kaldor, J. M.; Day, N. E.; Hemminki, K. *Eur. J. Cancer Clin. Oncol.* **1988**, *24*, 703.
6. Gourdie, T. A.; Valu, K. K.; Gravatt, G. L.; Boritzki, T. J.; Baguley, B. C.; Wakelin, L. P. G.; Wilson, W. R.; Woodgate, P. D.; Denny, W. A. *J. Med. Chem.* **1990**, *33*, 1177.
7. Fan, J.-Y.; Valu, K. K.; Woodgate, P. D.; Baguley, B. C.; Denny, W. A. *Anti-Cancer Drug Des.* **1997**, *12*, 181.
8. Wyatt, M. D.; Lee, M.; Hartley, J. A. *Anti-Cancer Drug Des.* **1997**, *12*, 49.
9. Bacherikov, V. A.; Chou, T.-C.; Dong, H.-J.; Zhang, X.; Chen, C.-H.; Lin, Y.-W.; Tsai, T.-J.; Lee, R.-Z.; Liu, L. F.; Su, T.-L. *Bioorg. Med. Chem.* **2005**, *13*, 3993.
10. Su, T.-L.; Lin, Y.-W.; Chou, T.-C.; Zhang, X.; Bacherikov, V. A.; Chen, C.-H.; Liu, L. F.; Tsai, T.-J. *J. Med. Chem.* **2006**, *49*, 3710.
11. Springer, C. J.; Dowell, R.; Burke, P. J.; Hadley, E.; Davies, D.; Blakey, D. C.; Melton, R. G.; Niculescu-Duvaz, I. *J. Med. Chem.* **1995**, *38*, 5051.
12. Springer, C. J.; Antoniw, P.; Bagshawe, K. D.; Searle, F.; Bisset, G. M. F.; Jarman, M. *J. Med. Chem.* **1990**, *33*, 677.
13. Springer, C. J. *Drugs Future* **1993**, *18*, 212.
14. Jordan, A. M.; Khan, T. H.; Malkin, H.; Helen, M. I.; Osborn, H. M. I. *Bioorg. Med. Chem.* **2002**, *10*, 2625.
15. Jordan, A. M.; Khan, T. H.; Osborn, H. M. I.; Photiou, A.; Riley, P. A. *Bioorg. Med. Chem.* **1999**, *7*, 1775.
16. Knaggs, S.; Malkin, H.; Osborn, H. M. I.; Williams, N. A. O.; Yaqoob, P. *Org. Biomol. Chem.* **2005**, *3*, 4002.
17. Su, T.-L.; Chou, T.-C.; Kim, J. Y.; Huang, J.-T.; Ciszewska, G.; Ren, W.-Y.; Otter, G. M.; Sirotnak, F. M.; Watanabe, K. A. *J. Med. Chem.* **1995**, *38*, 3226.
18. Su, T.-L. *Curr. Med. Chem.* **2002**, *9*, 1677.
19. Chang, J.-Y.; Lin, C.-F.; Pan, W.-Y.; Bacherikov, V.; Chou, T.-C.; Chen, C.-H.; Dong, H.; Cheng, S.-Y.; Tsai, T.-J.; Lin, Y.-W.; Chen, K.-T.; Chen, L.-T.; Su, T.-L. *Bioorg. Med. Chem.* **2003**, *11*, 4959.

20. Bacherikov, V. A.; Lin, Y. W.; Chang, J.-Y.; Chen, C. H.; Pan, W.-Y.; Dong, H.; Lee, R.-Z.; Chou, T.-C.; Su, T.-L. *Bioorg. Med. Chem.* **2005**, *13*, 6513.
21. Dowell, R. I.; Springer, C. J.; Davies, D. H.; Hadley, E. M.; Burke, P. J.; Boyle, F. T.; Melton, R. G.; Connors, T. A. *J. Med. Chem.* **1996**, *39*, 1100.
22. Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827.
23. Skehan, P.; Storeng, R. H.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenny, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
24. Chou, T.-C.; O'Connor, O. A.; Tong, W. P.; Guan, Y.-B.; Zhang, X.-G.; Stachel, S. J.; Lee, S.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8113.
25. Chou, T.-C., Martin, N. ComboSyn, Inc.: Paramus, NJ, 2005.
26. Chou, T.-C.; Talalay, P. *Adv. Enzyme Regul.* **1984**, *22*, 27.
27. Chou, T.-C. *Pharmacol. Rev.* **2006**, *58*, 621.
28. Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. *Exp. Cell Res.* **1988**, *175*, 184.
29. Friedmann, B. J.; Caplin, M.; Savic, B.; Shah, T.; Lord, C. J.; Ashworth, A.; Hartley, J. A.; Hochhauser, D. *Mol. Cancer Ther.* **2006**, *5*, 209.