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Design, synthesis, and biological evaluation of novel water-soluble *N*-mustards as potential anticancer agents

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ABSTRACT

A series of novel water-soluble *N*-mustard-benzene conjugates bearing a urea linker were synthesized. The benzene moiety contains various hydrophilic side chains are linked to the *meta*- or *para*-position of the urea linker via a carboxamide or an ether linkage. The preliminary antitumor studies revealed that these agents exhibited potent cytotoxicity in vitro and therapeutic efficacy against human tumor xenografts in vivo. Remarkably, complete tumor remission in nude mice bearing human breast carcinoma MX-1 xenograft and significant suppression against prostate adenocarcinoma PC3 xenograft were achieved by treating with compound **9aa′** at the maximum tolerable dose with relatively low toxicity. We also demonstrate that the newly synthesized compounds are able to induce DNA cross-linking through alkaline agarose gel shift assay. A pharmacokinetic profile of the representative **9aa′** in rats was also investigated. The current studies suggest that this agent is a promising candidate for preclinical studies.

1. Introduction

The bioavailability of drugs is one of the important factors to determine whether a drug can be successfully developed for clinical application. Consequently, the physicochemical properties, including solubility, permeability, stability pK_a, and lipophilicity/hydrophilicity balance, are key factors that influence the bioavailability of drugs. Compounds with poor solubility usually have a higher risk of failure during the period of new drug discovery and development because these properties may affect antitumor evaluations in animal models as well as pharmacokinetic and pharmacodynamic properties of the compound. Several approved drugs possess poor water solubility, resulting in reduced bioavailability. For example, the poor water-soluble paclitaxel (mitotic spindle inhibitor) when converted into its water-soluble poly(glutamic acid) conjugates shows some evidence for increased tumor availability of the drug for targeted therapy.[1–4] Similar efforts in developing water-soluble derivatives of camptothecin (DNA topoisomerase I inhibitors) led to the discovery of topotecan (**1**, Chart 1)[5] and irinotecan (CPT-11, **2**),[6] which have been approved for clinical use.

In one of our research projects on the discovery and development of new anticancer agents, we have synthesized a series of DNA-directed alkylating agents, in which the phenyl *N*-mustard pharmacophore (warhead) is linked to the DNA-affinic molecule (carrier, such as 9-anilinoacridines, acridines or quinolines) via a urea, carbamate or hydrazinecarboxamide linker.[7–9] These linkers were previously utilized for preparing antibody-directed enzyme prodrug therapy (ADEPT),[10] gene-directed enzyme prodrug therapy (GDEPT),[11] or melanocyte-directed enzyme prodrug therapy (MDEPT). It demonstrated that these linkers are able to reduce the chemical reactivity of the reactive *N*-mustard moiety.[12–14] We also reported that the *N*-mustard-DNA-affinic molecule conjugates exhibit potent antitumor activity against various human tumor xenograft models. These conjugates are able to induce more DNA interstrand cross-linking than other alkylating agents such as melphalan or cisplatin. However, these agents are generally lipophilic and have poor water-solubility, compromising antitumor and pharmacokinetic studies in animal models. To overcome the drawback of insufficient solubility of these agents, it is necessary to consider designing and synthesizing compounds with improved water-solubility during the period of discovery and development.

Earlier works on the developing water-soluble *N*-mustard derivatives by Denny and co-workers have introduced a variety of hydrophilic side chains to the benzene ring of 4-nitroaniline-*N*-mustards

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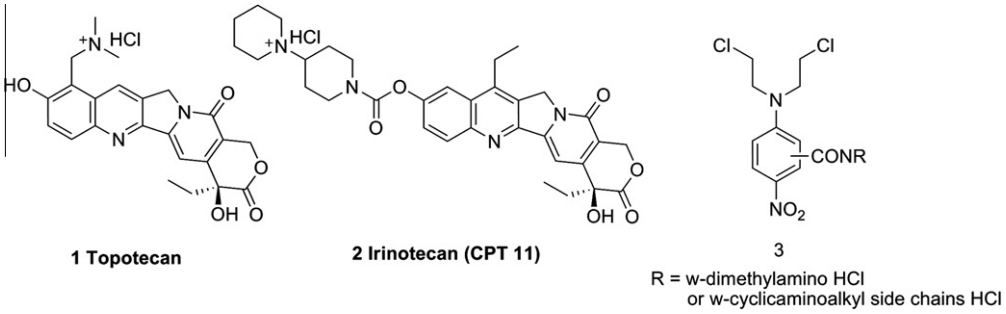
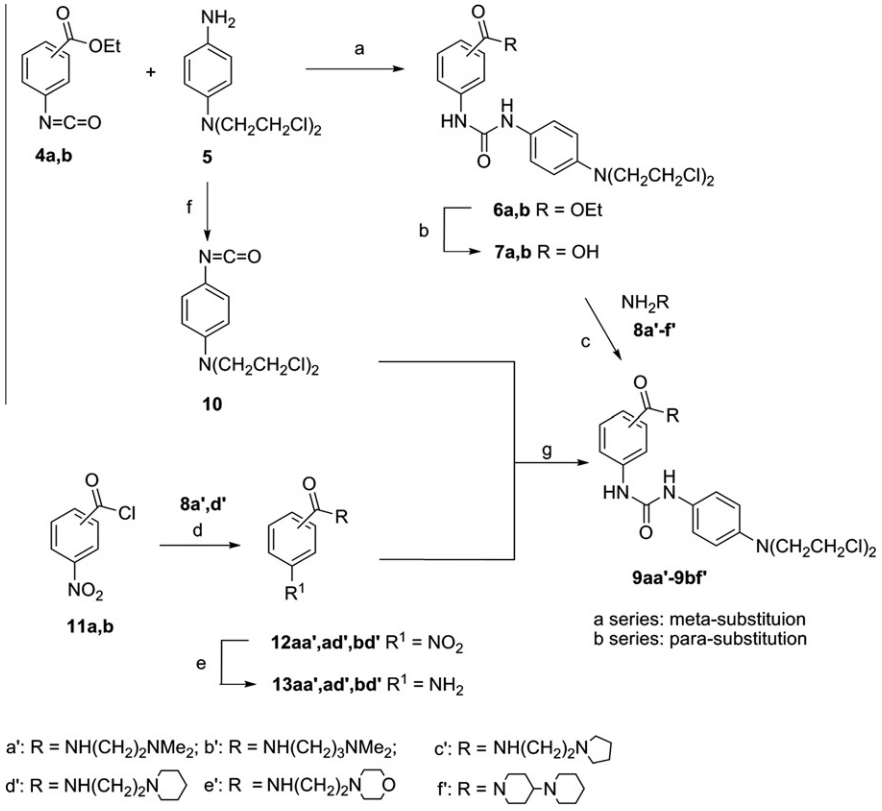


Chart 1.

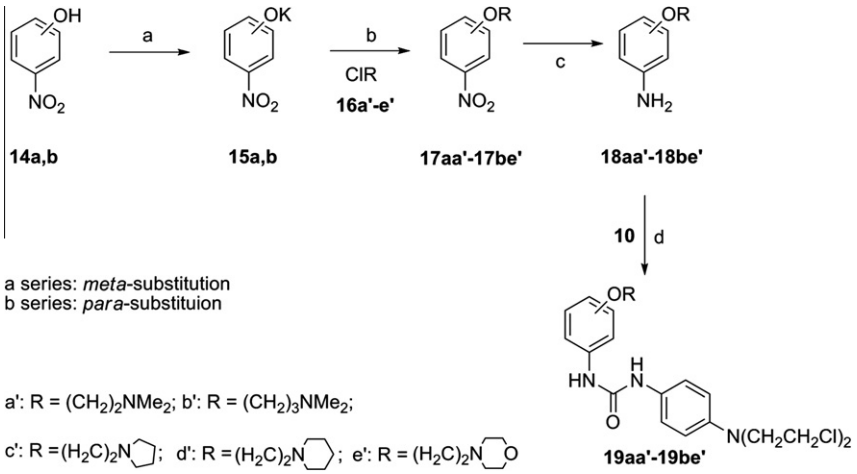
derivatives (**3**) via a carboxamide linker to form water-soluble bioreductive anticancer prodrugs.[15] Other studies also clearly demonstrated that linking a side chain containing a basic tertiary amino function can increase the aqueous solubility of drug by forming inorganic or organic acid salts.[16–20] These studies prompted us to design and synthesize chemical stable and water-soluble phenyl *N*-mustards, yet preserving the potent antitumor activity. We therefore prepared a series of water-soluble *N*-mustard-benzene conjugates via a urea linker. The benzene moiety contains a variety of water-soluble hydrophilic side chains including *N*,*N*-dimethylamino or cyclicamino functions, which can be converted into water-soluble derivatives by forming hydrochloride or other salts. The hydrophilic side chain in the benzene ring is located at the *para*- or *meta*-position to the urea linker via a carboxamide (compound **9aa′,bf′** series, Scheme 1) or an ether linkage (compound **19aa′,be′** series, Scheme 2). These conjugates were subjected to antitumor evaluation. The results show that they exhibit potent antitumor activity in inhibiting human tumor xenografts. The chemical synthesis, in vitro and in vivo antitumor activity as well as DNA cross-linking are described here in this paper.

2. Chemistry

The water-soluble phenyl *N*-mustard-benzene conjugates containing a hydrophilic carboxamide side-chain (**9a,b** series) were prepared starting from the commercially available ethyl 3- (or 4-)isocyanatobenzoate (**4a,b**) (Scheme 1). Thus, reactions of **4a,b** with the known *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**5**)[14] afforded **6a,b**. The products were hydrolyzed with concd HCl/AcOH (3:1 v/v) under reflux to yield benzoic acid derivatives **7a,b** following the literature procedure.[21] Coupling of **7a,b** with various amines (**8a′–8f′**) in dry DMF in the presence of DDC/HOBT/Et_3N afforded the desired compounds **9aa′–9bf′** in fair to good yields after purification by column chromatography. All final products obtained had poor water solubility, since they were in a free base form.



Scheme 1. Reagents and conditions: (a) Et_3N, THF, room temperature; (b) concd HCl/AcOH (3:1, v/v), reflux, 6 h; (c) DCC/HOBT, TEA, DMF, rt; (d) Et_3N, $CHCl_3$, rt; (e) 10% Pd/C/H_2, EAOAc; (f) triphosgene/$CHCl_3$/Et_3N, 0 °C; (g) Et_3N, DMF, rt.



Scheme 2. Reagents and conditions: (a) KOH/EtOH; (b) K_2CO_3/toluene, alkyl chlorides, reflux; (c) 10% Pd/C, H_2, 35 psi; (d) Et_3N/DMF, rt.

Since compound **9aa**′ was selected for further antitumor studies based on its cytotoxicity against tumor cell lines tested (see below), an alternative method was developed to optimize the synthesis of this agent (Scheme 1). The reaction of 3-nitrobenzoyl chloride (**11**) with *N*,*N*-dimethylethylamine hydrochloride (**8a**′) yielded benzoylcarboxamides **12**, which was then converted into the corresponding aniline derivatives **13** by catalytic hydrogenation (10% Pd/C, H_2) in EtOH. Reaction of **13** with 4-[*N*,*N*-bis(2-chloroethyl)amino]phenylisocyanate (**10**)[22] [freshly prepared by reacting **5** with triphosgene] in anhydrous DMF in the presence of triethylamine (TEA) afforded the desired **9aa**′ hydrochloride salt, which was found to be soluble in water. This synthetic method can be applied to preparing water-soluble **9aa**′–**9bf**′ hydrochloride or mesylate. For instance, we have synthesized **9ad**′ and **9bd**′ hydrochloride by following the same synthetic route as that for **9aa**′. However, one may obtain free compounds during purification by column chromatography if a long column and a large amount of silica gel is used. We have also prepared water-soluble mesylate salt of **9aa**′ by treating with methane sulfonic acid in MeOH.

The synthetic route for the preparation of phenyl *N*-mustard-benzene conjugates containing a hydrophilic ether side-chain (**19a,b** series) is depicted in Scheme 2. The reactions of the potassium salt of 3- (or 4-)nitrophenols (**15a** and **15b**, respectively, freshly prepared from the corresponding **14a,b** with KOH in EtOH) with various ω-aminoalkyl halides (**16a**′–**16e**′) in dry toluene afforded nitro substituted phenyl ether derivatives **17a,b** following the literature procedure.[23] The nitro function of **17a,b** was reduced to the corresponding aniline derivatives **18a,b** by catalytic hydrogenation (10% Pd/C/H_2). The products **18a,b** were then reacted with freshly prepared phenylisocyanate **10** in the presence of triethylamine to yield the final products **19aa**′–**19be**′. Similarly, the hydrochloride salt may be converted into its free form during purification by liquid column chromatography.

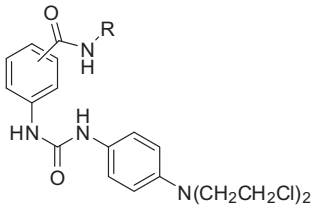
It should be noted that compounds **9aa**′–**9bf**′ were synthesized as a free base form as one can identify from their ^{1}H NMR spectrophotometer spectrum. For example, the protons of NMe_2 and NCH_2-functions in **9aa**′, **9ab**′, **9ba**′, and **9bb**′, which exist as a free base and have poor water-solubility, are located near δ 2.21 and 2.40, respectively. In contrast, the corresponding functions in **9aa**′ hydrochloride or mesylate salt, which are water-soluble, have a chemical shift at lower field, near δ 2.82 and 3.26, respectively. Similar observations were found in the series of **19aa**′–**19ae**′, in which the corresponding protons are located at higher fields (near δ 2.21 and 2.61, respectively), indicating that these agents exist in a free base form. The same protons in derivatives **19ba**′–**19be**′ have higher chemical shift values, demonstrating that they exist as hydrochloride salt. The assignment for whether compounds form a free base or salt is further confirmed by comparison with **17aa**′–**17be**′ and **18aa**′–**18be**′ (see ^{1}H NMR spectral data in Supplementary data). The results demonstrated that the ether linker located at the *para*-position to the urea moiety (**19ba**′–**19be**′) can easily form hydrochloride salts under the reaction conditions, but that has not happened in the corresponding *meta*-substituted derivatives (**19aa**′–**19ae**′). Although we are able to convert the free derivatives into its hydrochloride salts by dissolving compounds in a mixture of $CHCl_3$/MeOH followed with 20% HCl in ethyl acetate (data not shown), we surprisingly found that both free base (**19aa**′–**19ae**′) and hydrochloride salt forms (**19ba**′–**19be**′) have poor water-solubility.

3. Biological results and discussion

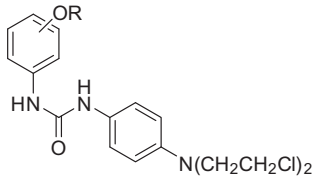
3.1. In vitro cytotoxicity

Human lymphoblastic leukemia (CCRF/CEM) and its drug-resistant sublines (CCRF-CEM/taxol and CCRF-CEM/VBL (330-fold resistant to taxol and 680-fold resistant to vinblastine, respectively) were used to evaluate the cytotoxicity of tested compounds and to realize whether they had multi-drug resistance (MDR) toward taxol or vinblastine in our antitumor screening program. The antiproliferative activities of the newly synthesized *N*-mustard-benzene conjugates are summarized in Table 1. The structure-activity relationship study shows that the newly synthesized compounds exhibit significant cytotoxicity with IC_{50} values of sub-micromolar range in inhibiting CCRF/CEM cell growth in culture. There is no significant difference in term of their cytotoxicities affected by the type of side-chain (carboxamide or ether linkage) and the location of the substituent (*meta*- or *para*-position). In general, compounds having an ether linkage are slightly more potent than the corresponding compounds bearing a carboxamide side-chain. Of this series of compounds, **9aa**′ is the most cytotoxic. In the series of compounds having a carboxamide side-chain, the tertiary amino substituent (ω-*N*,*N*-dimethylamino-, *N*-alkylpyrroliny- or *N*-alkylpiperidinyl function) does not have a big influence to their potency. However, the *N*-alkylmorpholinyl derivatives (**9ae**′ and **9be**′) are less cytotoxic against CCRF-CEM among the tested compounds. In the series of compounds having an ether linkage, derivatives having an ether side-chain located at the *para*-position to the urea linker are more cytotoxic than or as potent as the corresponding *meta*-substituted derivatives. Similarly, the *N*-alkylmorpholinyl derivatives (**19ae**′ and **19be**′) are slightly less cytotoxic against CCRF-CEM in comparison with other derivatives.

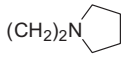
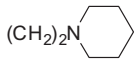
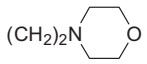
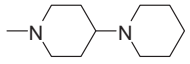
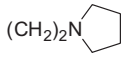
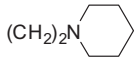
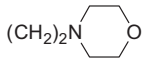
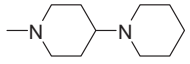
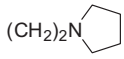
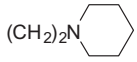
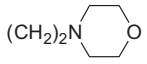
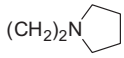
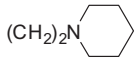
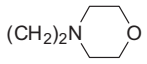
Table 1
The cytotoxicity of new *N*-mustards against human lymphoblastic leukemia (CCRF/CEM) and its drug-resistant sublines (CCRF-CEM/taxol and CCRF-CEM/VBL)[a]



9aa'-9bf'



19aa'-19be'

Compd	R	Cytotoxicity, IC_{50} (μM)		
		CCRF-CEM	CCRF-CEM/taxol[b]	CCRF-CEM/VBL[b]
9aa′	$(CH_2)_2NMe_2$	0.13 ± 0.002	1.22 ± 0.02 [9.4×][c]	0.80 ± 0.01 [6.2×]
9ab′	$(CH_2)_3NMe_2$	0.29 ± 0.012	21.20 ± 0.15 [9.4×]	44.03 ± 0.08 [151.8×]
9ac′		0.34 ± 0.001	25.20 ± 0.45 [73.7×]	36.56 ± 0.24 [106.9×]
9ad′		0.27 ± 0.02	4.62 ± 0.01 [17.0×]	10.49 ± 0.46 [43.7×]
9ae′		0.58 ± 0.002	8.16 ± 0.18 [14.1×]	16.29 ± 0.11 [28.1×]
9af′		0.28 ± 0.003	12.19 ± 0.66 [43.5×]	20.04 ± 0.48 [71.6×]
9ba′	$(CH_2)_2NMe_2$	0.23 ± 0.002	4.80 ± 0.07 [21.1×]	6.74 ± 0.29 [29.6×]
9bb′	$(CH_2)_3NMe_2$	0.26 ± 0.01	20.39 ± 0.80 [77.5×]	32.82 ± 0.34 [124.8×]
9bc′		0.27 ± 0.010	19.67 ± 0.45 [71.8×]	30.82 ± 0.34 [112.5×]
9bd′		0.36 ± 0.001	14.97 ± 1.47 [41.8×]	24.28 ± 0.29 [67.8×]
9be′		0.73 ± 0.01	19.82 ± 0.19 [27.1×]	29.76 ± 0.26 [16.8×]
9bf′		0.22 ± 0.01	20.47 ± 0.66 [91.4×]	32.18 ± 0.12 [143.7×]
19aa′	$(CH_2)_2NMe_2$	1.32 ± 0.001	0.81 ± 0.001 [0.6×]	1.48 ± 0.003 [1.1×]
19ab′	$(CH_2)_3NMe_2$	0.32 ± 0.01	2.05 ± 0.01 [6.4×]	2.37 ± 0.35 [7.4×]
19ac′		0.35 ± 0.01	1.20 ± 0.002 [3.5×]	4.20 ± 0.01 [12.1×]
19ad′		0.19 ± 0.003	0.57 ± 0.02 [3.0×]	0.73 ± 0.01 [3.8×]
19ae′		0.41 ± 0.10	0.73 ± 0.07 [1.8×]	1.13 ± 0.58 [2.8×]
19ba′	$(CH_2)_2NMe_2$	0.10 ± 0.002	1.50 ± 0.01 [7.6×]	1.19 ± 0.04 [6.02×]
19bb′	$(CH_2)_3NMe_2$	0.12 ± 0.001	1.36 ± 0.001 [11.8×]	1.26 ± 0.001 [10.9×]
19bc′		0.14 ± 0.001	1.57 ± 0.001 [11.5×]	2.05 ± 0.10 [15.0×]
19bd′		0.12 ± 0.003	0.50 ± 0.002 [4.02×]	0.72 ± 0.03 [7.47×]
19be′		0.35 ± 0.01	0.84 ± 0.01 [2.4×]	0.64 ± 0.01 [1.8×]
Taxol	—	0.003 ± 0.0001	0.43 ± 0.04 [330×]	1.27 ± 0.05 [980×]
Vinblastine	—	0.0007 ± 0.0001	0.08 ± 0.01 [106.2×]	0.50 ± 0.12 [679.5×]

[a] Cell growth inhibition was measured by the XTT assay[26] for leukemic cells and the SRB assay[27] for solid tumor cells after 72-h incubation using a microplate spectrophotometer as described previously.[28] Similar in vitro results were obtained by using the Cell Counting Kit-8 for the CCK-8 assays as described by technical manual of Dojindo Molecular Technologies, Inc. (Gaithersburg, MD; website: www.dojindo.com). 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[30] based on the median-effect principle and plot using the serial deletion analysis.[31,32] 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.

We investigated whether the newly synthesized compounds are multidrug resistant to the distinct drugs, such as taxol or vinblastin. The in vitro cytotoxicity of these derivatives against CCRF-CEM/taxol and CCRF-CEM/VBL reveal that they generally have no or little cross-resistance to these two natural products except compounds **9ab′**, **9ac′**, **9af′**, **9bb′**, **9bc′**, and **9bf′**, which have certain extent of cross-resistance (Table 1). It also demonstrates that compounds having an ether side-chain (compound **19a,b** series)

Table 2
Cytotoxicity of new *N*-mustards against human solid tumors (H1299, CL1–0, CL1–5, PC3, HCT-116, MX-1 and MCF-7) cell growth in vitro

Compd	Cytotoxicity, IC_{50} (μM)						
	H1299[a]	CL1–0 [a]	CL1–5 [a]	PC3 [a]	HCT-116 [b]	MX-1 [b]	MCF-7 [a]
9aa′	10.37 ± 2.67	14.59 ± 2.01	6.08 ± 1.01	1.88 ± 0.73	0.69 ± 0.03	0.57 ± 0.02	0.63 ± 0.11
9ab′	ND	ND	ND	ND	0.85 ± 0.01	2.33 ± 0.05	ND
9ac′	ND	ND	ND	ND	0.47 ± 0.001	2.17 ± 0.01	ND
9ad′	6.22 ± 1.91	8.67 ± 1.02	4.64 ± 1.43	1.70 ± 0.67	0.31 ± 0.002	0.73 ± 0.004	1.18 ± 0.95
9ae′	ND	ND	ND	ND	1.21 ± 0.072	1.45 ± 0.01	ND
9af′	11.47 ± 2.99	6.23 ± 1.67	8.09 ± 1.49	3.19 ± 0.99	0.81 ± 0.072	2.24 ± 0.01	3.09 ± 1.01
9ba′	ND	ND	ND	ND	0.39 ± 0.002	1.09 ± 0.001	ND
9bb′	12.70 ± 2.99	16.2 ± 4.32	9.79 ± 2.45	5.25 ± 1.77	4.54 ± 0.02	1.11 ± 0.023	5.17 ± 1.91
9bc′	ND	ND	ND	ND	0.36 ± 0.01	2.33 ± 0.07	ND
9bd′	ND	ND	ND	ND	0.91 ± 0.02	4.40 ± 0.05	ND
9be′	ND	ND	ND	ND	2.74 ± 0.001	3.39 ± 0.16	ND
9bf′	9.63 ± 2.01	15.84 ± 3.10	8.51 ± 1.56	1.91 ± 0.99	0.64 ± 0.01	3.64 ± 0.01	1.54 ± 0.85
19ad′	1.52 ± 0.79	2.73 ± 0.91	3.25 ± 1.01	1.13 ± 0.89	0.27 ± 0.01	0.19 ± 0.01	1.82 ± 0.95
19ba′	4.38 ± 1.06	4.07 ± 1.59	3.01 ± 0.53	1.67 ± 0.73	0.27 ± 0.001	0.28 ± 0.003	1.25 ± 0.99
19bb′	9.79 ± 1.56	7.09 ± 1.42	5.11 ± 1.34	0.94 ± 0.23	0.29 ± 0.001	0.26 ± 0.01	1.32 ± 0.32
19bc′	2.25 ± 0.88	3.01 ± 0.94	5.92 ± 1.56	1.78 ± 0.29	0.70 ± 0.0002	0.49 ± 0.01	1.84 ± 0.77
19bd′	1.84 ± 0.91	2.87 ± 0.68	4.75 ± 1.12	0.93 ± 0.14	0.27 ± 0.001	0.15 ± 0.01	1.24 ± 0.39

[a] Cell growth inhibition was determined by the Alamar blue assay[29] in a 72 h incubation using a microplate spectrophotometer as described previously.
[b] Cell growth inhibition was measured by the SRB assay[27] for solid tumor cells after 72-h incubation using a microplate spectrophotometer as described previously.[28]

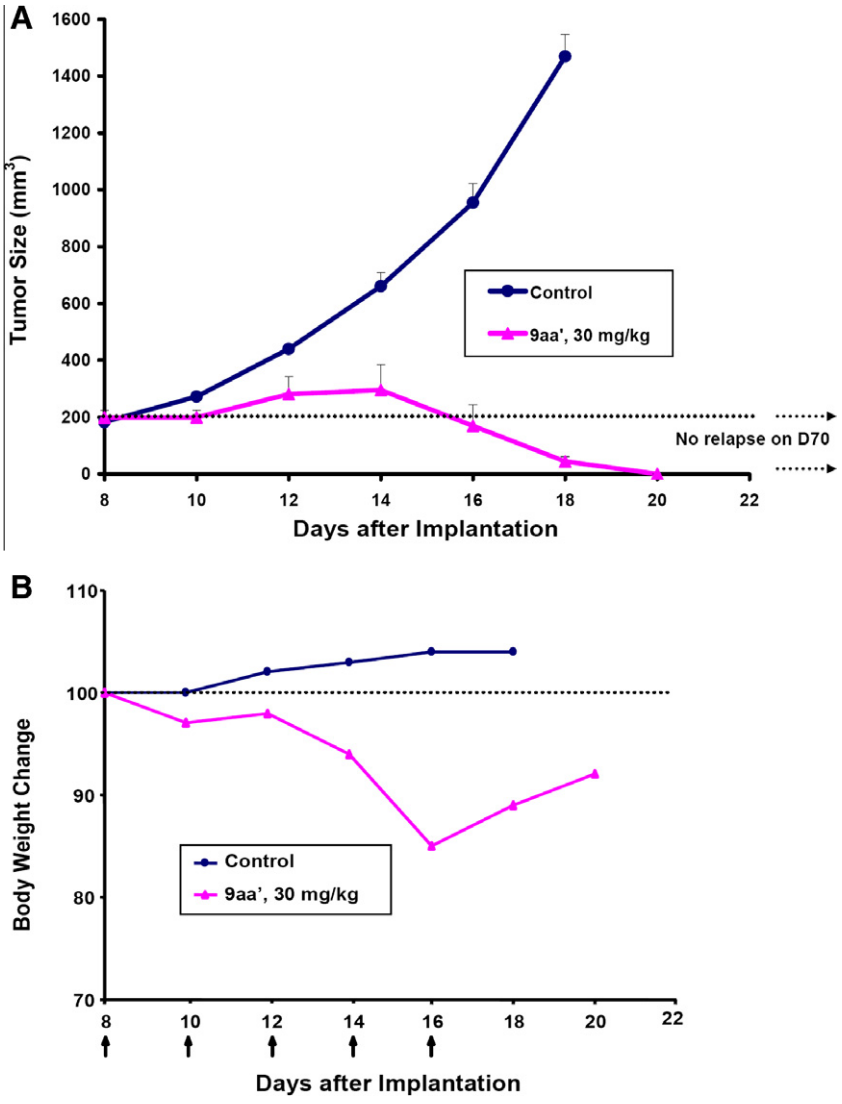


Figure 1. Therapeutic effect of **9aa′** in nude mice bearing human breast carcinoma MX-1 xenograft (30 mg/kg, Q2D × 5, iv injection, $n = 5$); Treatment started on Day 8 after tumor implantation. All treatments were carried out via iv injection. CR is referred as complete tumor remission. (A) Average tumor size changes; (B) average body weight changes.

have less MDR in comparison with the corresponding derivatives bearing a carboxamide side-chain (compound **9a,b** series).

To further explore the antiproliferative activity of the new synthesized *N*-mustards, the selected compounds were studied for their cytotoxicity in inhibiting other human solid tumors such as human non-small cell lung cancer (H1299), lung adenocarcinoma (CL1–0 and CL1–5), colon cancer (HCT-116), prostate cancer (PC3) and human breast tumor (MX-1 and MCF-7) cell growth in vitro (Table 2). The results showed that these agents, in general, are more cytotoxic toward human prostate, colon and breast tumors among the tumor cell lines tested. It is of great interest to note that the tested compounds have almost equal potency against both MX-1 and resistant MCF-7 cell lines in vitro. Of these agents, compounds **19ad**′ and **19bd**′ are shown to have a broad spectrum of antitumor activity against the tested tumor cell growth in culture.

3.2. In vivo therapeutic effect

In the present study, we evaluated the antitumor activity of compound **9aa**′ since this agent shows to be the most cytotoxic against solid tumor cell lines tested. Figure 1 shows the therapeutic efficacy of this agent in nude mice bearing human adenocarcinoma MX-1 xenograft. The complete tumor remission (CR) was achieved at the maximum tolerable doses of 30 mg/kg (Q2D × 5, intravenous injection), and maintained for over

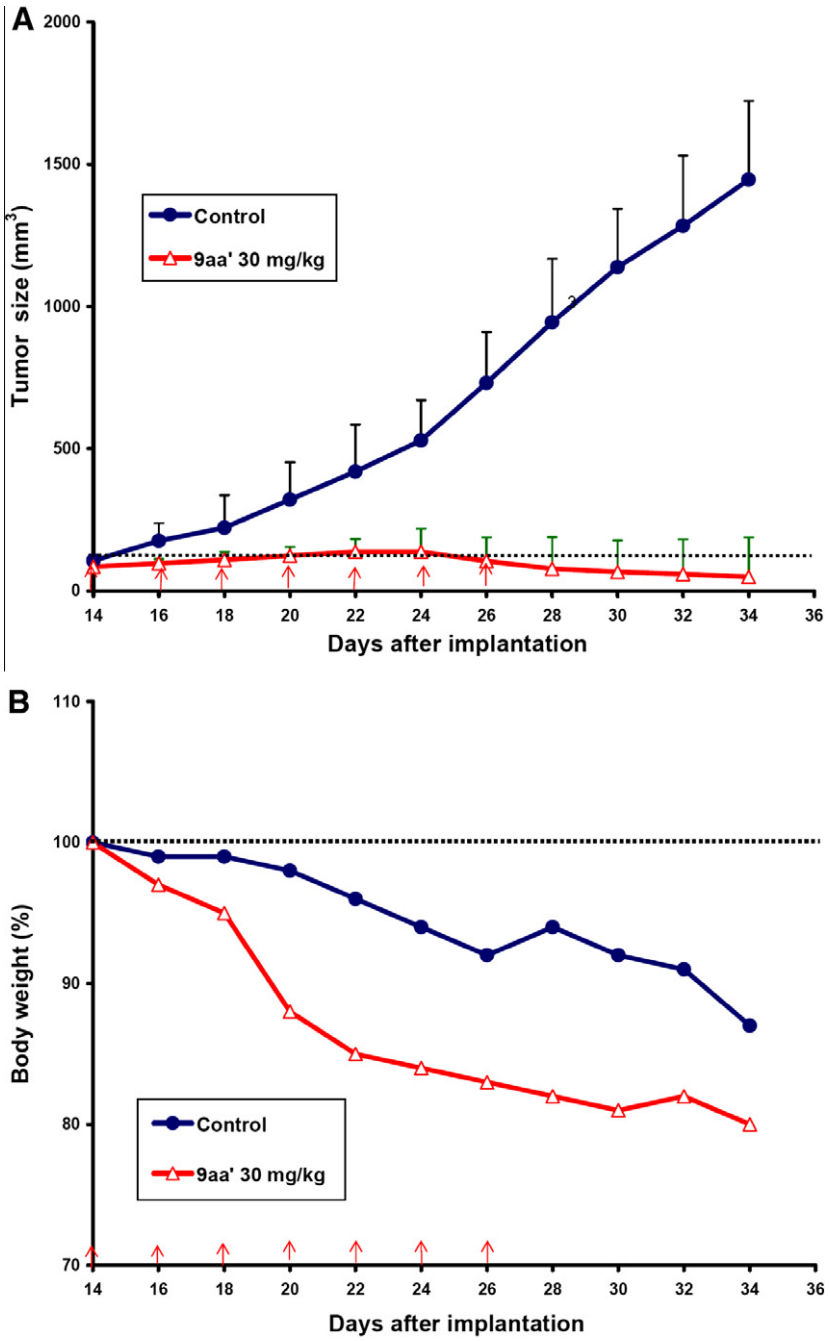


Figure 2. Therapeutic effect of **9aa**′ in nude mice bearing human prostate cancer PC3 xenograft (30 mg/kg (Q2D × 4), followed by 40 mg/kg (Q2D × 3), iv injection $n = 4$). (A) Average tumor size changes; (B) average body weight changes. Note that for PC3 xenograft, tumor-growth led to body weight decreases in the control experiment.

70 days. The therapeutic effect of compound **9aa**′ was further investigated against human prostate carcinoma PC3 xenograft in nude mice. As shown in Figure 2, more than 99% of tumor suppression was observed at the dose of 30 mg/kg (Q2D × 4), followed by 40 mg/kg (Q2D × 3). We have also evaluated the antitumor activity of compound **9aa**′ in nude mice bearing human colon cancer HCT-116 xenograft (Fig. 3). More than 95% tumor suppression was observed at the dose of 30 mg/kg (Q2D × 5, iv injection). In comparison, the antitumor effects of **9aa**′ (30 mg/kg, Q2D × 6, iv injection $n = 4$, where n = number of mice tested per experiment) with cyclophosphamide (80 mg/kg, Q2D × 6, iv injection) in nude mice bearing human glioma U87 MG xenograft (Fig. 4) revealed that the former compound was more potent, but less toxic than that of the latter drug. In all in vivo xenograft experiments, body weight is referred to total body weight minus tumor weight assuming 1 mm^3 = 1 mg.

3.3. DNA cross-linking study

To realize whether the newly synthesized compounds are capable of cross-linking with DNA double strands, pEGFP-N1 DNA was reacted with compounds, **9aa**′, **9ad**′, **19bd**′, and **19bb**′ at various concentrations as indicated (1, 5, and 10 μM) and subjected to alkaline agarose gel shifting assay after BamH1 digestion (Fig. 5).[24] Melphalan was used as the positive control. As shown in Fig. 5, all the tested compounds were able to induce DNA interstrand cross-linking, suggesting that DNA cross-linking may be the main mechanism of action for these agents.

3.4. Cell cycle inhibition

Previous studies demonstrated that DNA-interacting agents damage DNA, inducing G2 arrest in the cell cycle through the G2

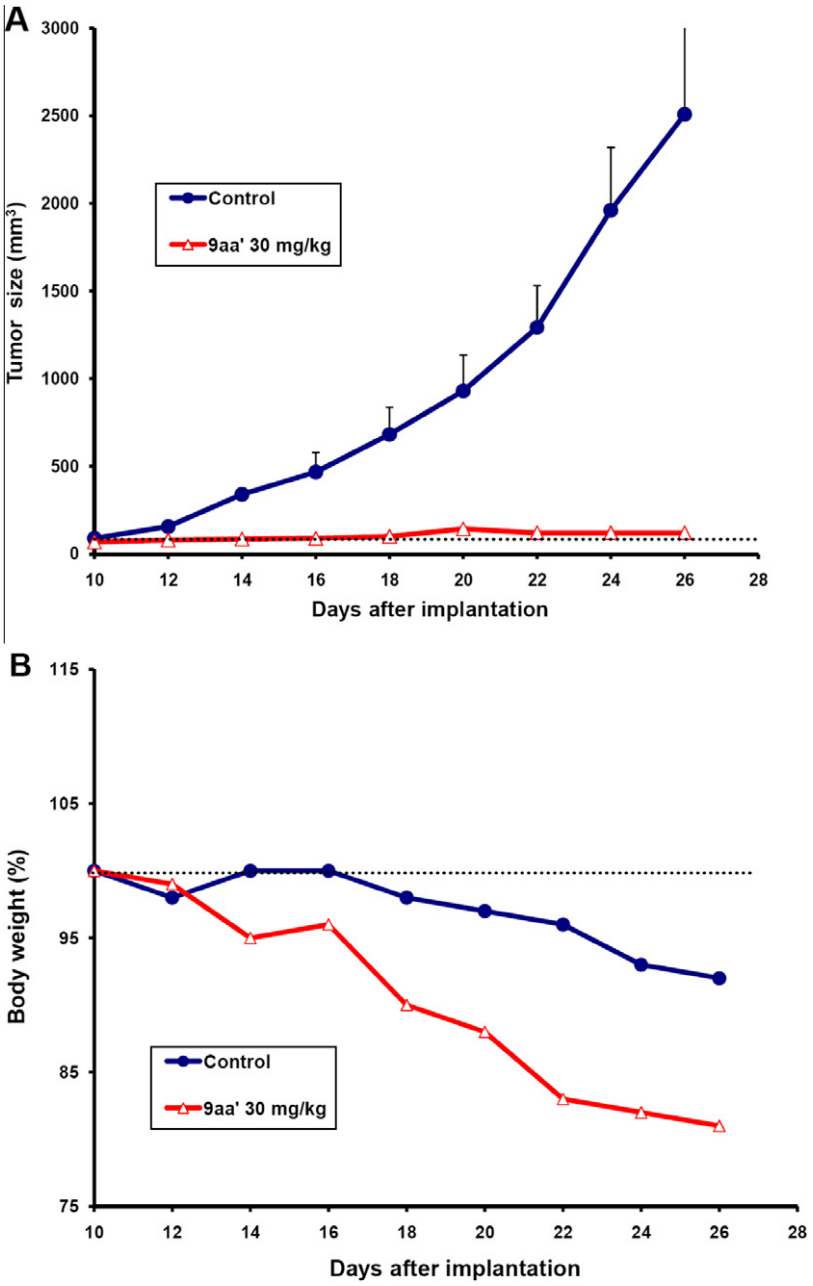


Figure 3. Therapeutic effects of **9aa**′ in nude mice bearing HCT-116 xenograft (30 mg/kg (Q2D × 5, iv injection $n = 4$). (A) Average tumor size changes; (B) average body weight changes. Note that for HCT-116 xenograft, tumor-growth led to body weight decreases in the control experiment.

DNA-damage checkpoint pathway.[25] We studied the inhibitory effect of **9aa**′ on cell cycle distribution (Table 3). The human non-small lung carcinoma H1299 cells were treated with **9aa**′ at the concentrations of 2.5, 5, and 10 μM for 24 h. The cells were harvested, stained with propidium iodide (PI) and analyzed with a flow cytometer. As shown in Table 3, **9aa**′ treatment induced significant G2/M arrest in H1299 cells. Similar G2/M arrest was previously observed in SW626 cells treated with melphalan.[26] Furthermore, we also found that increased sub-G1 populations were noticed in H1299 cells treated with **9aa**′ (Table 3).

3.5. Pharmacokinetic profile of 9aa′

Prior to clinical studies, our leading compound **9aa**′ were subjected to pharmacokinetic studies in healthy male Sprague Dawley rats. A single intravenous dose was administered via an indwelling catheter in jugular vein to a group of two male rats at a dose level of 1.0 mg/kg. The formulations were prepared as a solution in 5.0% w/v DMSO with 10% w/v Cremophor in di-distilled water. Serial blood samples were collected from jugular vein catheter up to 24 h post-dose from all animals in Group. Concentration levels of **9aa**′ were determined in plasma using a validated LC–MS/MS assay with a lower limit of quantification (LLOQ) of 2.5 ng/mL. The plasma concentration-time data above the LLOQ at each dose level were used in the calculation of pharmacokinetic parameters of **9aa**′ using the validated program WinNonlin™, version 5.2.1. Pharmacokinetic parameters are summarized in Table 4. The result showed that the mean terminal half-life ($t_{1/2}$) and mean residence time (MRT) of **9aa**′ were 0.58 h and 0.11 h, respectively. The mean apparent plasma

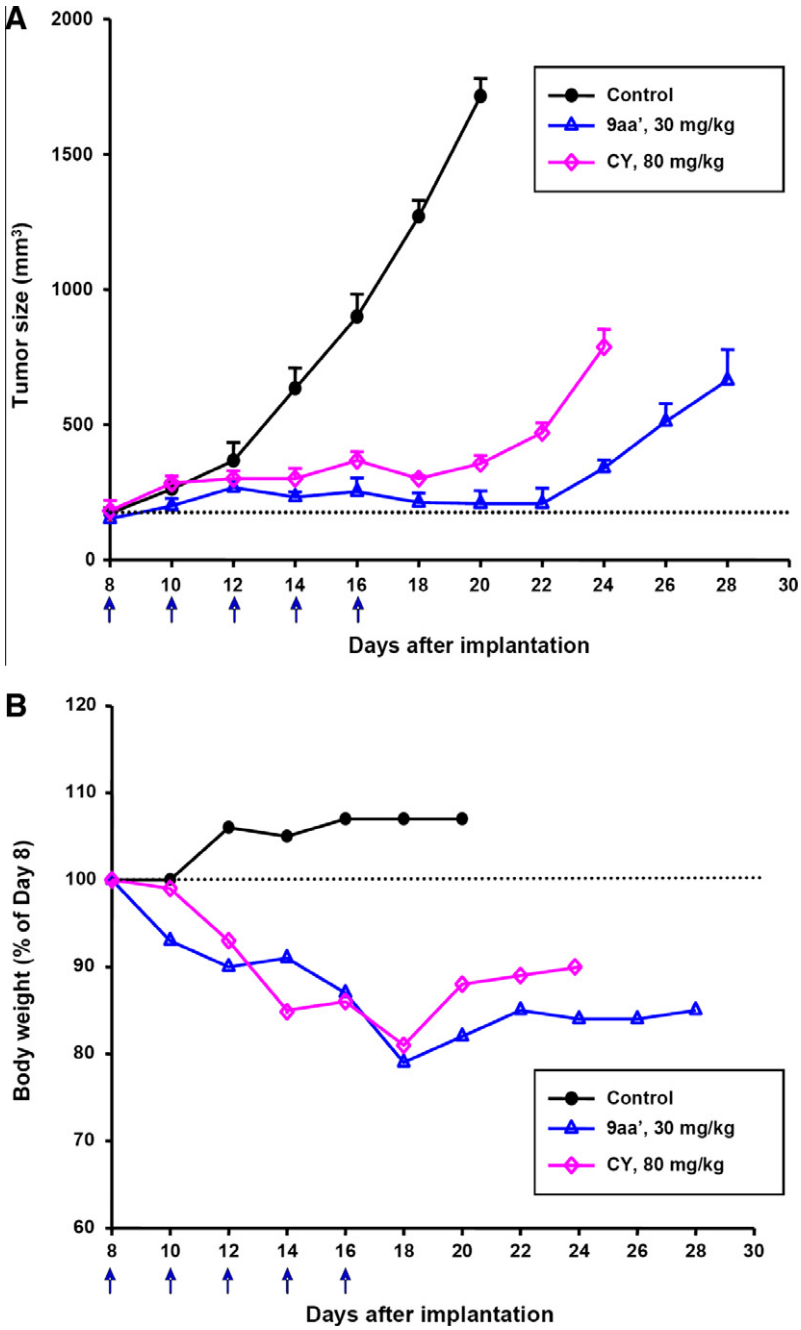


Figure 4. Therapeutic effect of **9aa**′ (30 mg/kg, Q2D × 6, iv injection $n = 4$) and cyclophosphamide (80 mg/kg, Q2D × 6, iv injection $n = 4$) in nude mice bearing human glioma U87 MG xenograft. (A) Average tumor size changes; (B) average body weight changes.

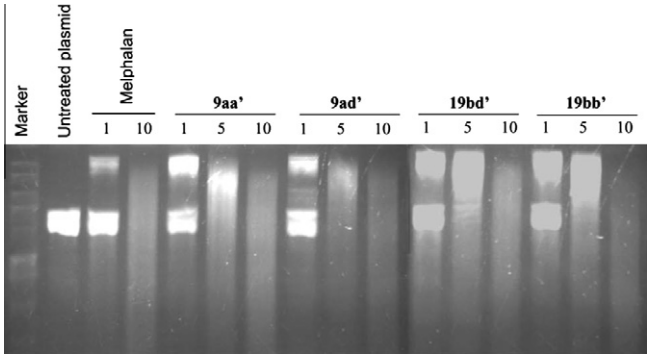


Figure 5. Representative DNA cross-linking gel shift assay for **9aa**′, **9ad**′, **19bd**′, and **19bb**′ at various concentrations as indicated. Control lane shows single stranded DNA (SL), while cross-linking (CL) shown in all tested lanes is DNA double-stranded cross-linking. Melphalan (1 and 10 μM) was used as a positive control.

clearance of **9aa**′ was 18.0 mL/min/kg with the apparent volume of distribution at steady state of 0.15 L/kg.

4. Conclusion

In the present studies, we have designed and synthesized a series of water-soluble DNA alkylating *N*-mustard derivatives, in which the phenyl *N*-mustard pharmacophore is attached to a benzene ring via a urea linker. We have demonstrated that these conjugates can be easily converted into water-soluble derivatives by forming hydrochloride or mesylate salts. The newly synthesized compounds exhibited significant antitumor activity both in vitro and in vivo against various human tumor xenografts. Detailed structure-activity relation studies revealed that the types of the hydrophilic side-chain (carboxamide or ether) linked to the benzene ring does not greatly affect their cytotoxicities. Moreover, we showed that these derivatives have little or no cross-resistance to either taxol or vinblastine. Among the newly synthesized derivatives, we selected compound **9aa**′ for further in vivo antitumor evaluation since this agent shows to be the most cytotoxic against solid tumor cell lines tested. Remarkably, complete tumor remission in nude mice bearing human breast carcinoma MX-1 xenograft and significant suppression against prostate adenocarcinoma PC3 xenograft were achieved with acceptable toxicity. We also demonstrate that the newly synthesized compounds are able to induce DNA cross-linking by alkaline agarose gel shift assay. Furthermore, the pharmacokinetic study reveals that **9aa**′ has a good pK_a profile in rats with a half-life of 0.58 h. The present studies suggest that this agent is a promising candidate for preclinical studies.

5. Experimental section

5.1. Chemistry: general methods

All commercial chemicals and solvents were reagent grade and were used without further purification unless otherwise specified. 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. All reported yields are isolated yields after chromatography or crystallization. Elemental analyses were done on a Heraeus CHN-O Rapid instrument. ^{1}H NMR spectra were recorded on a 600 MHz, Brucker AVANCE 600 DRX and 400 MHz, Brucker Top-Spin spectrometers in the indicated solvent. The chemical shifts were reported in ppm (δ) relative to TMS. High performance liquid chromatography analyses for checking purity of synthesized compounds were recorded on a Hitachi D-2000 Elite instrument: column, Mightysil RP-18 GP 250-4.6 (5 μm); mobile phase, 90% A, 5% B, and 5% C in 25 min (mobile phase A = acetonitrile, B = THF, and C = H_2O); flow rate, 1 mL/min; injected sample 10 μL, column temp, 27 °C; wavelength, 254 nm. The purity of all compounds was ⩾95% based on analytical HPLC.

5.2. Synthesis of water-soluble *N*-mustards having a carboxamide linker (9aa′,bf′ series)

5.2.1. Ethyl 3-[3-(4-(bis(2-chloroethyl)amino)phenyl)ureido]-benzoate (6a)

To a suspension of *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (5, 6.12 g, 20 mmol) in dry chloroform (100 mL)

Table 3
Cell cycle inhibition in human non-small cell lung adenocarcinoma H1299 by treating with compound **9aa**′

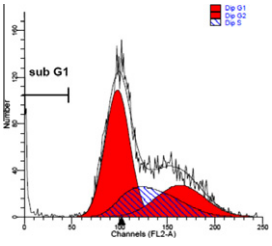
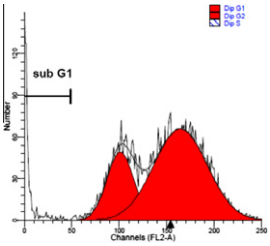
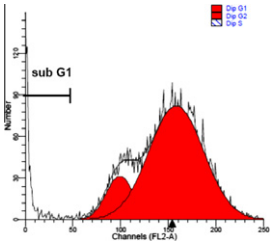
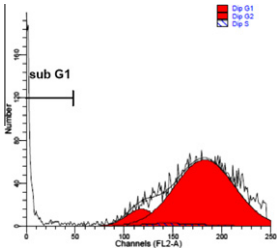
Concentration (μM)	0	2.5	5	10
				
Sub-G1	14.1 ± 0.7	20.0 ± 4.9	19.7 ± 4.5	26.0 ± 4.7
G1	42.9 ± 0.8	2.3 ± 1.0	10.7 ± 3.9	6.5 ± 3.2
S	22.5 ± 0.3	1.8 ± 2.6	8.8 ± 1.4	5.2 ± 4.6
G2/M	20.5 ± 0.9	55.9 ± 3.6	60.8 ± 2.5	62.3 ± 1.5

Table 4
Summary of pharmacokinetic parameters of **9aa**′ following intravenous injection to rats (n = 2)

Dose (mg/kg)	C_0 (ng/mL)	$t_{1/2}$ (h)	MRT (h)	$AUC_{(0-last)}$ (h ng/mL)	$AUC_{(0-\infty)}$ (h ng/mL)	CL_{ss} (mL/min/kg)	V_{ss} (L/kg)
1	11,980	0.58	0.11	925.81	934.93	18.00	0.15

was added TEA (3.5 mL) at −10 °C. A solution of ethyl 3-isocyanatobenzoate (**4a**, 3.32 mL, 20 mmol) in dry chloroform was added dropwise to the above mixture at the same temperature. The reaction mixture was then allowed to stir at room temperature for 12 h. The reaction mixture was evaporated reduced pressure. The product was purified by column chromatography using CH_2Cl_2/MeOH (100:2, v/v) as an eluent. The fraction containing the main product were combined and evaporated to dryness. The white solid residue was triturated with hexane, collected by filtration, and dried to give **6a**, yield: 6.81 g (80%); mp 164–165 °C; ^{1}H NMR (DMSO-d_6) δ 1.32 (3H, t, J = 7.2 Hz, Me), 3.67–3.72 (8H, m, 4 × CH_2), 4.31 (2H, q, J = 7.2 Hz, CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.28 (2H, d, J = 8.8 Hz, 2 × ArH), 7.40 (1H, t, J = 7.2 Hz, ArH), 7.53 (1H, d, J = 8.0 Hz, ArH), 7.63–7.65 (1H, m, ArH), 8.12 (1H, s, ArH), 8.34 and 8.80 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{20}H_{23}Cl_2N_3O_3$: C, H, N.

5.2.2. Ethyl 4-[3-(4-(bis(2-chloroethyl)amino)phenyl)ureido]-benzoate (6b)

Compound **3b** was synthesized by following the same procedure as that for **3a** and prepared from *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**5**, 7.65 g, 25 mmol) and ethyl 4-isocyanatobenzoate (**4b**, 4.78 g, 25 mmol). Yield 8.11 g (76%); mp 221–223 °C; ^{1}H NMR (DMSO-d_6) δ 1.30 (3H, t, J = 7.2 Hz, Me), 3.70–3.71 (8H, m, 4 × CH_2), 4.27 (2H, q, J = 7.2 Hz, CH_2), 6.72 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.56 (2H, d, J = 8.8 Hz, 2 × ArH), 7.86 (2H, d, J = 8.8 Hz, 2 × ArH), 8.46 and 8.96 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{20}H_{23}Cl_2N_3O_3$: C, H, N.

5.2.3. 3-[3-(4-(Bis(2-chloroethyl)amino)phenyl)ureido]benzoic acid (7a)

A solution of **6a** (4.253 g, 10 mmol) in mixture of concd HCl/AcOH (100 mL, 3:2 v/v) was heated at 100 °C for 6 h. The reaction mixture was cooled to room temperature, the separated white solid was collected by filtration, the solid cake was washed with water and dried to give **7a**; yield: 3.52 g (89%); mp 191–192 °C; ^{1}H NMR (DMSO-d_6) δ 3.70 (8H, s, 4 × CH_2), 6.74 (2H, d, J = 8.8 Hz, 2 × ArH), 7.30 (2H, d, J = 8.8 Hz, 2 × ArH), 7.30 (1H, t, J = 8.0 Hz, ArH), 7.51 (1H, d, J = 7.6 Hz, ArH), 7.63–7.65 (1H, m, ArH), 8.09 (1H, s, ArH), 8.09 and 8.82 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{18}H_{19}Cl_2N_3O_3{\cdot}2H_2O$: C, H, N.

5.2.4. 4-[3-(4-(Bis(2-chloroethyl)amino)phenyl)ureido]benzoic acid (7b)[21]

Compound **4b** was synthesized by following the same procedure as that for **7a** and was prepared from 6**b** (2.13 g, 5 mmol) in a mixture of conc. HCl/AcOH (70 mL, 3:2 v/v). Yield 1.78 g (90%); mp 238–241 °C (reported >300 °C, decomp.);[21] ^{1}H NMR (DMSO-d_6) δ 3.69–3.73 (8H, m, 4 × CH_2), 6.74 (2H, d, J = 8.8 Hz, 2 × ArH), 7.30 (2H, d, J = 8.8 Hz, 2 × ArH), 7.54 (2H, d, J = 8.8 Hz, 2 × ArH), 7.83 (2H, d, J = 8.8 Hz, 2 × ArH), 8.88 and 9.39 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{18}H_{19}Cl_2N_3O_3{\cdot}2H_2O$: C, H, N.

5.2.5. 1-[3-((2-(Dimethylamino)ethyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)-phenyl]urea hydrochloride (9aa′)

A mixture of **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and *N*,*N*-dimethylethylenediamine (**8a′**, 0.264 g, 3 mmol) in dry DMF (25 mL) was stirred at room temperature for 72 h. The reaction mixture was filtered to remove the by-product urea and the filtrate was evaporated in vacuo to dryness. The residue was dissolved in CH_2Cl_2 (250 mL) and successively washed with water (100 mL), satd $NaHCO_3$ aqueous solution (100 mL) and brine (100 mL), dried over Na_2SO_4 and filtered. The filtrate was evaporated to dryness and the residue was chromatographed over a silica gel column using CH_2Cl_2/MeOH (100:9) as an eluent. The fractions containing the main product were combined and evaporated in vacuo to dryness. The residue was crystallized from ethyl acetate to give white solid. The solid was suspended in ethyl acetate (50 mL) and treated with 2.5 M HCl in ethyl acetate (5.0 mL) at 0 °C. The excess solvent was evaporated to dryness and solid separated was dried in vacuo to give desired product **9aa′**, 580 mg (58%); mp 156–157 °C; ^{1}H NMR (DMSO-d_6) δ 2.20 (6H, s, 2 × NMe), 2.42 (2H, t, J = 6.8 Hz, CH_2), 3.32–3.35 (2H, m, CH_2), 3.68–3.70 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.34–7.39 (2H, m, 2 × ArH), 7.59 (1H, d, J = 7.6 Hz, ArH), 7.83 (1H, s, ArH), 8.29 (1H, t, J = 5.2 Hz, exchangeable, CONH), 8.40 and 8.72 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{22}H_{29}Cl_2N_5O_2$: C, H, N.

By following the same procedure as described for **9aa′**, the following compounds were synthesized.

5.2.6. 1-[3-((3-(Dimethylamino)propyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)-phenyl]urea (9ab′)

Compound **9ab′** was prepared from **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and *N*,*N*-dimethylpropane-1,3-diamine (**8b′**, 0.305 g, 3 mmol). Yield, 645 mg (65%); mp 138–140 °C; ^{1}H NMR (DMSO-d_6) δ 1.61–1.66 (2H, m, CH_2), 2.13 (6H, s, 2 × NMe), 2.25 (2H, t, J = 6.8 Hz, CH_2), 3.27 (2H, q, J = 6.8 Hz, CH_2), 3.66–3.72 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.32–7.38 (2H, m, 2 × ArH), 7.58 (1H, d, J = 8.0 Hz, ArH), 7.84 (1H, s, ArH), 8.40 and 8.72 (each 1H, br s, exchangeable, 2 × NH), 8.46 (1H, t, J = 5.2 Hz, exchangeable, CONH). Anal. Calcd for $C_{23}H_{31}Cl_2N_5O_2{\cdot}2H_2O$: C, H, N.

5.2.7. 1-[3-((2-(Pyrrolidin-1-yl)ethyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)-phenyl]urea hydrochloride (9ac′)

Compound **6ac′** was prepared from **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and 1-(2-aminoethyl)pyrolidine (**8c′**, 0.341 g, 3 mmol). Yield, 591 mg (56%); mp 129–131 °C; ^{1}H NMR (DMSO-d_6) δ 1.87–1.91 (5H, m, CH), 3.0 (2H, t, J = 5.2 Hz, CH_2), 3.32 (2H, d, J = 6.0 Hz, CH_2), 3.62–3.64 (3H, m, CH), 3.72 (8H, s, 4 × CH_2), 6.73 (2H, d, J = 8.8 Hz, 2 × ArH), 7.30 (2H, d, J = 8.8 Hz, 2 × ArH), 7.34–7.36 (2H, m, 2 × ArH), 7.62 (1H, d, J = 9.2 Hz, ArH), 7.94 (1H, s, ArH), 8.78–8.79 (1H, m, exchangeable, CONH), 9.0 and 9.33 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{31}Cl_2N_5O_2{\cdot}2H_2O$: C, H, N.

5.2.8. 1-[3-((2-(Piperidin-1-yl)ethyl)carbamoyl)phenyl]-3-(4-(bis(2-chloroethyl)amino)-phenyl)urea hydrochloride (9ad′)

Compound **9ad′** was prepared from **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and 1-(2-aminoethyl)piperidine (**8d′**, 0.384 g). Yield, 683 mg (63%); mp 151–152 °C; ^{1}H NMR (DMSO-d_6) δ 1.38–1.51 (7H, m, CH), 2.41–2.45 (5H, m, CH), 3.34–3.38 (2H, m, CH), 3.66–3.72 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.32–7.38 (2H, m, 2 × ArH), 7.58 (1H, d, J = 7.6 Hz, ArH), 7.84 (1H, s, ArH), 8.28 (1H, t, J = 5.2 Hz, exchangeable, CONH), 8.40 and 8.70 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{25}H_{33}Cl_2N_5O_2$: C, H, N.

5.2.9. 1-[3-((2-Morpholinoethyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)phenyl]- urea hydrochloride (9ae′)

Compound **9ae′** was prepared from **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and 1-(2-aminoethyl)morpholine (**8e′**, 0.260 g, 3 mmol). Yield, 588 mg (54%); mp 167–169 °C; ^{1}H NMR (DMSO-d_6) δ 2.41–2.47 (5H, m, CH), 3.36–3.38 (2H, m, CH), 3.57 (5H, br s, CH), 3.69 (8H, s,

4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.33–7.40 (2H, m, 2 × ArH), 7.59 (1H, d, J = 7.2 Hz, ArH), 7.91 (1H, s, ArH), 8.30 (1H, t, J = 5.6 Hz, exchangeable, CONH), 8.34 and 8.66 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{31}Cl_2N_5O_3$: C, H, N.

5.2.10. 1-[4-(Bis(2-chloroethyl)amino)phenyl]-3-[3-(4-(piperidin-1-yl)piperidine-1-carbonyl] phenyl)urea hydrochloride (9af′)

Compound **6af′** was prepared from **7a** (0.792 g, 2 mmol), DCC (0.618 g, 3 mmol), HOBT (0.505 g, 3 mmol), TEA (0.4 mL) and 4-piperidino-piperidine (**8f′**, 0.504 g, 3 mmol). Yield, 700 mg (60%); mp 118–119 °C; ^{1}H NMR (DMSO-d_6) δ 1.37–1.48 (8H, m, CH), 1.68–1.79 (2H, m, CH), 2.47–2.50 (3H, m, CH), 2.73 (1H, br s, CH), 2.98 (1H, br s, CH), 3.37 (4H, br s, CH), 3.66–3.72 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 6.90–6.92 (1H, d, J = 7.2 Hz, ArH) 7.27 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29–7.32 (1H, m, ArH), 7.41 (1H, d, J = 8.0 Hz, ArH), 7.51 (1H, s, ArH), 8.42 and 8.71 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{28}H_{37}Cl_2N_5O_2$: C, H, N.

5.2.11. 1-[4-((2-(Dimethylamino)ethyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)-phenyl]urea hydrochloride (9ba′)

Compound **9ba′** was prepared from **7b** (0.396 g, 1 mmol), DCC (0.309 g, 1.5 mmol), HOBT (0.202 g, 1.5 mmol), TEA (0.2 mL) and **8a′**, (0.132 g, 1.5 mmol). Yield, 340 mg (72%); mp 189–190 °C; ^{1}H NMR (DMSO-d_6) δ 2.17 (6H, s, 2 × NMe), 2.38 (2H, br s, CH_2), 3.28 (2H, br s, CH_2), 3.69 (8H, br s, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.49 (2H, d, J = 8.8 Hz, 2 × ArH), 7.76 (2H, d, J = 8.8 Hz, 2 × ArH), 8.18, 8.47 and 8.83 (each 1H, br s, exchangeable, 3 × NH). Anal. Calcd for $C_{22}H_{29}Cl_2N_5O_2$: C, H, N.

5.2.12. 1-[4-((3-(Dimethylamino)propyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino)-phenyl]urea (9bb′)

Compound **9bb′** was prepared from **7b** (0.594 g, 1.5 mmol), DCC (0.464 g, 2.25 mmol), HOBT (0.304 g, 2.25 mmol), TEA (0.3 mL) and **8b′** (0.229 g, 2.25 mmol). Yield, 534 mg (69%); mp 203–205 °C; ^{1}H NMR (DMSO-d_6) δ 1.62–1.67 (2H, m, CH_2), 2.13 (6H, s, 2 × NMe), 2.25 (2H, t, J = 6.8 Hz, CH_2), 3.24 (2H, q, J = 6.8 Hz, CH_2), 3.69–3.72 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.49 (2H, d, J = 8.8 Hz, 2 × ArH), 7.75 (2H, d, J = 8.8 Hz, 2 × ArH), 8.39 (1H, t, J = 5.2 Hz, exchangeable, CONH), 8.49 and 8.86 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{23}H_{31}Cl_2N_5O_2{\cdot}2H_2O$: C, H, N.

5.2.13. 1-[4-((2-(Pyrrolidin-1-yl)ethyl)carbamoyl)phenyl]-3-[4-(bis(2-chloroethyl)amino) phenyl]urea hydrochloride (9bc′)

Compound **9bc′** was prepared from **7b** (0.594 g, 1.5 mmol), DCC (0.464 g, 2.25 mmol), HOBT (0.304 g, 2.25 mmol), TEA (0.3 mL) and **8c′** (0.256 g, 2.25 mmol). Yield, 419 mg (53%); mp 183–185 °C; ^{1}H NMR (DMSO-d_6) δ 1.05–2.00 (4H, m, CH), 3.02–3.04 (2H, m, CH), 3.30–3.33 (2H, m, CH), 3.42–3.45 (4H, m, CH), 3.70 (8H, s, 4 × CH_2), 6.72 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.52 (2H, d, J = 8.8 Hz, 2 × ArH), 7.85 (2H, d, J = 8.8 Hz, 2 × ArH), 8.67–8.69 (1H, m, exchangeable, CONH), 9.01 and 9.48 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{31}Cl_2N_5O_2{\cdot}2H_2O$: C, H, N.

5.2.14. 1-[4-((2-(Piperidin-1-yl)ethyl)carbamoyl)phenyl]-3-(4-(bis(2-chloroethyl)amino)-phenyl)urea hydrochloride (9bd′)

Compound **9bd′** was prepared from **7b** (0.594 g, 1.5 mmol), DCC (0.464 g, 2.25 mmol), HOBT (0.304 g, 2.25 mmol), TEA (0.3 mL) and **8d′** (0.288 g, 2.25 mmol). Yield, 400 mg (49%); mp 198–200 °C; ^{1}H NMR (DMSO-d_6) δ 1.37–1.39 (1H, m, CH), 1.67–1.79 (5H, m, CH), 2.98 (2H, s, CH), 3.43–3.50 (4H, m, CH), 3.60–3.63 (2H, m, CH_2), 3.67–3.71 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.49 (2H, d, J = 8.8 Hz, 2 × ArH), 7.75 (2H, d, J = 8.8 Hz, 2 × ArH), 8.30–8.31 (1H, m, exchangeable, CONH), 8.35 and 8.66 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{25}H_{33}Cl_2N_5O_2$: C, H, N.

5.2.15. 1-[4-((2-Morpholinoethyl)carbamoyl)phenyl]-3-[4-(bis-(2-chloroethyl)amino)phenyl]- urea hydrochloride (9be′)

Compound **9be′** was prepared from **7b** (0.594 g, 1.5 mmol), DCC (0.464 g, 2.25 mmol), HOBT (0.304 g, 2.25 mmol), TEA (0.3 mL) and **8e′** (0.195 g, 2.25 mmol). Yield, 538 mg (61%); mp 234–235 °C; ^{1}H NMR (DMSO-d_6) δ 2.41–2.47 (6H, m, CH), 3.35–3.39 (2H, m, CH), 3.56–3.57 (4H, m, CH), 3.70–3.71 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.28 (2H, d, J = 8.8 Hz, 2 × ArH), 7.49 (2H, d, J = 8.8 Hz, 2 × ArH), 7.75 (2H, d, J = 8.8 Hz, 2 × ArH), 8.20–8.23 (1H, m, exchangeable, CONH), 8.42 and 8.79 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{31}Cl_2N_5O_3$: C, H, N.

5.2.16. 1-[4-(Bis(2-chloroethyl)amino)phenyl]-3-[4-(4-(piperidin-1-yl)piperidine-1-carbonyl]phenyl)urea hydrochloride (9bf′)

Compound **9bf′** was prepared from **7b** (0.594 g, 1.5 mmol), DCC (0.464 g, 2.25 mmol), HOBT (0.304 g, 2.25 mmol), TEA (0.3 mL) and **8f′** (0.378 g, 2.25 mmol). Yield, 638 mg (73%); mp 222–224 °C; ^{1}H NMR (DMSO-d_6) δ 1.38–1.50 (9H, m, CH), 1.75 (2H, br s, CH), 2.51 (2H, br s, CH), 2.84 (2H, br s, CH), 3.32–3.40 (4H, m, CH), 3.68–3.72 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.27–7.31 (4H, m, 4 × ArH), 7.48 (2H, d, J = 8.8 Hz, 2 × ArH), 8.46 and 8.81 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{28}H_{37}Cl_2N_5O_2{\cdot}0.8H_2O$: C, H, N.

5.3. Alternative method for preparing 9aa′, 9ad′, and 9bd′ hydrochloride

5.3.1. *N*-(2-(Dimethylamino)ethyl)-3-nitrobenzamide (12aa′)

A solution of *N*,*N*-dimethylethylene diamine (**8a′**, 13.2 mL) in dry THF was added to a solution of 3-nitrobenzoyl chloride (**11a**, 18.5 g, 100 mmol) in dry THF at 0 °C. After being stirred at room temperature for 3 h, the solvent was evaporated to dryness. The oily residue was dissolved in minimum amount of water and then evaporated to dryness. The solid separated was recrystallized from water and washed with hexane to give **12aa′**, 21 g, (89%); mp 89–90 °C; MS (ESI) *m/z*: 238 [M+H]$^+$. ^{1}H NMR (DMSO-d_6) δ 2.19 (6H, s, 2 × NMe), 2.43 (2H, t, J = 6.8 Hz, CH_2), 3.40 (2H, q, J = 12.6 and 6.4 Hz, CH_2), 7.78 (1H, t, J = 7.9 Hz, ArH), 8.29 (1H, d, J = 7.8 Hz, ArH), 8.37–8.39 (1H, m, ArH), 8.68 (1H, s, ArH), 8.79–8.82 (1H, m, exchangeable, NH).

By following the same procedure as described for **12aa′**, the following compounds were synthesized.

5.3.2. 3-Nitro-*N*-(2-(piperidin-1-yl)ethyl)benzamide (12ad′)

Compound **12ad′** was synthesized from 1-(2-aminoethyl)piperidine (**8d′**, 3.31 g, 22.0 mmol) and 3-nitrobenzoyl chloride (**11ad′**, 4.0 g, 21.5 mmol). Yield, 5.5 g, (92%); mp 203–204 °C; MS (ESI) *m/z*: 278 [M+H]$^+$. ^{1}H NMR (DMSO-d_6) δ 1.67–1.69 (6H, m, 3 × CH_2), 2.91–2.92 (2H, m, CH_2), 3.22–3.32 (2H, m, CH_2), 3.50–3.70 (2H, m, CH_2), 3.72–3.74 (2H, m, CH_2), 7.76–7.80 (1H, m, ArH), 8.37–8.43 (2H, m, 2 × ArH), 8.72–8.73 (1H, m, ArH), 9.38 (1H, t, J = 5.4 Hz, exchangeable, NH).

5.3.3. 4-Nitro-*N*-(2-(piperidin-1-yl)ethyl)benzamide (12bd′)

Compound **12bd′** was synthesized from 2-(piperidin-1-yl)ethanamine (**8d′**, 4.23 g, 33 mmol) and 3-nitrobenzoyl chloride (**11b**, 5.56 g, 30 mmol). Yield, 7.2 g, (86%); mp 165–167 °C (lit.[27] 97–99 °C). MS (ESI) *m/z*: 278 [M+H]$^+$. ^{1}H NMR (DMSO-d_6) δ 1.54–1.57 (2H, m, CH), 1.81–1.82 (4H, m, CH), 3.09–3.22 (4H, m, CH),

3.24–3.27 (2H, m, CH), 3.72–3.76 (2H, m, CH), 8.21 (2H, d, J = 8.7 Hz, 2 × ArH), 8.32 (2H, d, J = 8.7 Hz, 2 × ArH), 9.40 (1H, t, J = 5.2 Hz, exchangeable, NH).

5.3.4. 3-Amino-*N*-(2-(dimethylamino)ethyl)benzamide (13aa′)

10% Palladium on charcoal (1.5 g) was suspended in a solution of **12aa′** (10.6 g, 4.5 mmol) in ethyl acetate. The mixture was hydrogenated at 35 psi for 3 h. The reaction mixture was filtered through a pad of Celite and the filtrate was evaporated in vacuo to dryness to give **13aa′**, 8.62 g (93%); mp 97–100 °C. ^{1}H NMR (DMSO-d_6) δ 2.16 (6H, s, 2 × NMe), 2.36 (2H, t, J = 6.8 Hz, CH_2), 3.40 (2H, q, J = 13 and 6.5 Hz, CH_2), 5.20 (1H, br s, exchangeable, NH), 6.66 (1H, d, J = 7.8 Hz, ArH), 6.91 (1H, d, J = 7.7 Hz, ArH), 7.00 (1H, s, ArH), 7.05 (1H, t, J = 7.4 Hz, ArH), 8.05–8.08 (1H, m, exchangeable, NH). The product pure enough and was used directly for the next reaction without further purification.

By following the same procedure as described for **13aa′**, the compounds **13ad′** and **13bd′** were synthesized.

5.3.5. 3-Amino-*N*-(2-(piperidin-1-yl)ethyl)benzamide (13ad′)

Compound **13ad′** was synthesized from **12ad′** (3.0 g, 10 mmol) and Pd/C (0.5 g). Yield was 2.0 g (75%). ^{1}H NMR (DMSO-d_6) δ 1.67–1.82 (6H, m, 3 × CH_2), 2.88–2.90 (2H, m, CH_2), 3.15–3.30 (2H, m, CH_2), 3.50–3.64 (2H, m, CH_2), 3.66–3.69 (2H, m, CH_2), 4.20 (2H, br s, exchangeable, NH_2), 7.20 (1H, d, J = 7.8 Hz, ArH), 7.37 (1H, t, J = 7.8 Hz, ArH), 7.54 (1H, s, ArH), 7.62 (1H, d, J = 7.8 Hz, ArH), 8.96 (1H, t, J = 5.4 Hz, exchangeable, NH). The product pure enough and was used directly for the next reaction without further purification.

5.3.6. 4-Amino-*N*-(2-(piperidin-1-yl)ethyl)benzamide (13bd′)

Compound **13ad′** was synthesized from **12bd′** (10.6 g, 4.5 mmol) and Pd/C (1.5 g). Yield was 8.62 g (93%); mp 109–110 °C (lit.[25] 118–120 °C). ^{1}H NMR (DMSO-d_6) δ 1.52 (2H, m, CH), 1.76 (4H, m, CH), 3.07 (6H, m, CH), 3.58–3.59 (2H, m, CH), 5.67 (2H, s, exchangeable, NH_2), 6.54 (2H, d, J = 8.4 Hz, 2 × ArH), 7.64 (2H, d, J = 8.4 Hz, 2 × ArH), 8.43 (1H, s, exchangeable, NH). The product pure enough and was used directly for the next reaction without further purification.

5.3.7. Compound 9aa′ hydrochloride

A solution of known 4-[*N*,*N*-bis(2-chloroethyl)-amino]phenyl-isocyanate (**10**) [freshly prepared from *N*,*N*-bis(2-chloroethyl)-benzene-1,4-diamine hydrochloride (**5**, 2.68 g, 8.75 mmol) by treating with triphosgene (1 g, 3.4 mmol) in the presence of TEA (1.25 mL) at −10 °C] in dry DMF was added dropwise to a solution of **13aa′** (1.22 g, 5 mmol) in dry DMF containing Et_3N (1.5 mL) at room temperature. After being stirred at room temperature for 8 h, the solvent was evaporated to dryness in vacuo. The residue was purified by column chromatography using $CHCl_3$/MeOH (v/v 100:5) as the eluent. The fraction containing main products were combined and evaporated to dryness to give water-soluble **9aa′** hydrochloride, 1.5 g (78%); mp 172–173 °C; ^{1}H NMR (DMSO-d_6) δ ^{1}H NMR (DMSO-d_6) δ 2.82 (6H, s, 2 × NMe), 3.26 (2H, t, J = 5.8 Hz, CH_2), 3.62–3.65 (2H, m, CH_2), 3.66–3.71 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.8 Hz, 2 × ArH), 7.29 (2H, d, J = 8.8 Hz, 2 × ArH), 7.32–7.37 (1H, m, ArH), 7.47 (1H, d, J = 7.6 Hz, ArH), 7.61 (1H, d, J = 8.4 Hz, ArH), 7.95 (1H, s, ArH), 8.74 (1H, t, J = 5.2 Hz, exchangeable, CONH), 8.95 and 9.26 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{22}H_{29}Cl_2N_5O_2$·HCl·H_2O: C, H, N.

5.3.8. Compound 9aa′ mesylate

A solution of **9aa′** hydrochloride (900 mg) in H_2O (500 mL) was neutralized with saturated $NaHCO_3$ aqueous solution to pH 7–8 and then extracted with $CHCl_3$ (300 mL × 3). The organic layer was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure. The residue (800 mg, 1.55 mmol) was dissolved in EtOH (200 mL). Methanesulfonic acid (149 mg, 155 mmol) was also added to the solution. The mixture was stirred at room temperature for 3 h and the solvent was removed under reduced pressure. The solid residue was recrystallized from EtOH to yield **9aa′** mesylate, 645 mg 68%, mp 155–156 °C; ^{1}H NMR (DMSO) δ 2.36 (3H, s, CH_3SO_3), 2.85 (3H, s, NMe), 2.86 (3H, s, NMe), 3.27 (2H, m, CH_2), 3.60 (2H, m, CH_2), 3.69 (8H, m, 4 × CH_2) 6.71 (2H, d, J = 9.0 Hz, 2 × ArH), 7.30 (2H, d, J = 9.0 Hz, 2 × ArH), 7.37 (1H, d, J = 7.7 Hz, ArH), 7.43 (1H, d, J = 7.7 Hz, ArH), 7.56 (1H, d, J = 7.7 Hz, ArH), 8.0 (1H, s, ArH), 8.49 (1H, s, NH), 8.62 (1H, br, exchangeable, NH), 8.81 (1H, s, NH), 9.27 (1H, br, NH). Anal. Calcd for $C_{23}H_{33}N_5O_5Cl_2S$·0.25H_2O: C, H, N, S.

By following the same procedure as described for **9aa′** hydrochloride, the compounds **9ad′** hydrochloride and **9bd′** hydrochloride were synthesized.

5.3.9. Compound 9ad′ hydrochloride

Compound **9ad′** was prepared from **13ad′** (1.5 g, 6 mmol) and *N*-mustard isocyanate **10** [freshly prepared from **5** (4.0 g, 13 mmol) and triphosgene (2.58 g, 9.5 mmol)]. Yield, 1.4 g (46%); mp 98–99; ^{1}H NMR (DMSO-d_6) δ 1.76–1.78 (6H, m, 3 × CH_2), 2.90–2.91 (2H, m, CH_2), 3.18–3.20 (2H, m, CH_2), 3.44–3.50 (2H, m, CH_2), 3.67–3.68 (2H, m, CH_2), 3.69–3.71 (8H, m, 4 × CH_2), 6.71 (2H, d, J = 8.1 Hz, 2 × ArH), 7.29 (2H, d, J = 8.1 Hz, 2 × ArH), 7.34 (1H, t, J =7.8 Hz, ArH), 7.46 (1H, d, J = 7.5 Hz, ArH), 7.60 (1H, d, J = 7.8 Hz, ArH), 7.93 (1H, s, ArH), 8.76 (1H, br s, exchangeable, NH), 8.84, 9.15 (each 1H, s, exchangeable, 2 × NH). HRMS (FAB$^+$) *m/z* calcd for $C_{25}H_{33}Cl_2N_5O_2$ [M+H]$^+$: 506.4678; found: 506.2072.

5.3.10. Compound 9bd′ hydrochloride

Compound **9bd′** was prepared from **13bd′** (0.50 g, 2 mmol) and *N*-mustard isocyanate **10** [freshly prepared from **5** (1.07 g, 3.5 mmol) and triphosgene (0.4 g, 1.36 mmol)]. Yield, 0.66 g (64%); mp 127–128; ^{1}H NMR (DMSO-d_6) δ 1.36–1.41 (1H, m, CH), 1.68–1.79 (5H, m, CH), 2.87–2.96 (2H, m, CH), 3.20–3.23 (2H, m, CH), 3.51–3.54 (2H, m, CH), 3.63–3.66 (2H, m, CH), 3.67–3.72 (8H, m, 4 × CH_2), 6.73 (2H, d, J = 8.9 Hz, 2 × ArH), 7.30 (2H, d, J = 8.9 Hz, 2 × ArH), 7.53 (2H, d, J = 8.6 Hz, 2 × ArH), 7.85 (2H, d, J = 8.6 Hz, 2 × ArH), 8.75 (1H, t, J = 5.3 Hz, exchangeable, CONH), 9.01 and 9.48 (each 1H, s, exchangeable, 2 × NH). HRMS (FAB$^+$) *m/z* calcd for $C_{25}H_{33}Cl_2N_5O_2$ [M+H]$^+$: 506.4678; found: 506.2076.

5.4. Synthesis of water-soluble *N*-mustards having an ether linker (19aa′–19be′ series)

Detailed procedures for the synthesis of intermediates **15a,b**, **17aa′–17be′**, and **18aa′–18be′** along with their spectroscopic data are provided in the Supplementary data.

5.4.1. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-(2-(dimethylamino)ethoxy)phenyl)urea (19aa′)

Triethylamine (5 mL) was added dropwise to a solution of phenylisocyanate **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.24 g, 4.2 mmol) in dry $CHCl_3$ (100 mL)] at −10 °C and stirred for 30 min. A solution of **18aa′** (1.384 g, 6 mmol) in $CHCl_3$ (25 mL) containing TEA (3 mL) at −10 °C was added slowly to the above solution. The reaction mixture was then allowed to stir at room temperature for 16 h and the reaction mixture was washed successively with water (350 mL) and brine (350 mL). The organic layer was dried (Na_2SO_4) and filtered. The filtrate was evaporated in vacuo to dryness and the residue was chromatographed on a silica gel column using CH_2Cl_2/MeOH (100:6) as an eluent. The fractions containing the main product were combined and evaporated to dryness. The white solid residue was triturated

with hexane and then collected by filtration to give **19aa′**, 0.710 g (46%); mp 143–145 °C; ^{1}H NMR (DMSO-d_6) δ 2.21 (6H, s, 2 × NMe), 2.61 (2H, s, CH_2), 3.69 (8H, br s, 4 × CH_2), 4.00 (2H, s, CH_2), 6.51–6.53 (1H, m, ArH), 6.70–6.72 (2H, m, 2 × ArH), 6.88–6.90 (1H, m, ArH), 7.13–7.18 (2H, m, 2 × ArH), 7.26–7.28 (2H, m, 2 × ArH), 8.32 and 8.52 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{21}H_{28}Cl_2N_4O_2$: C, H, N.

By following the same procedure as described for **19aa′**, the following compounds were prepared.

5.4.2. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-(3-(dimethylamino)propoxy)phenyl)urea (19ab′)

Compound **19ab′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18ab′** (1.384 g, 6 mmol). Yield, 1.247 g (43%); mp 127–128 °C; ^{1}H NMR (DMSO-d_6) δ 1.84–1.89 (2H, m, CH_2), 2.21 (6H, s, 2 × NMe), 2.44 (2H, t, J = 7.2 Hz, CH_2), 3.69–3.70 (8H, m, 4 × CH_2), 3.95 (2H, t, J = 7.2 Hz, CH_2), 6.48–6.51 (1H, m, ArH), 6.70 (2H, d, J = 8.8 Hz, 2 × ArH), 6.86 (1H, d, J = 8.0 Hz, ArH), 7.13 (1H, t, J = 8.0 Hz, ArH), 7.20 (1H, s, ArH), 7.27 (2H, d, J = 8.8 Hz, 2 × ArH), 8.40 and 8.62 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{22}H_{30}Cl_2N_4O_2{\cdot}0.6H_2O$: C, H, N.

5.4.3. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-(2-(pyrrolidin-1-yl)ethoxy)phenyl)urea (19ac′)

Compound **19ac′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18ac′** (1.456 g, 6 mmol). Yield, 1.36 g (45%); mp 124–125 °C; ^{1}H NMR (DMSO-d_6) δ 1.68 (4H, s, 2 × CH_2), 2.50 (4H, s, 2 × CH_2), 2.77 (2H, s, CH_2), 3.69 (8H, s, 4 × CH_2), 4.01 (2H, s, CH_2), 6.51 (1H, d, J = 7.2 Hz, ArH), 6.70 (2H, d, J = 8.0 Hz, 2 × ArH), 6.88 (1H, d, J = 7.6 Hz, ArH), 7.13 (1H, t, J = 8.0 Hz, ArH), 7.18 (1H, s, ArH), 7.27 (2H, d, J = 8.0 Hz, 2 × ArH), 8.37 and 8.57 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{23}H_{30}Cl_2N_4O_2$: C, H, N.

5.4.4. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-(2-(piperidin-1-yl)ethoxy)phenyl)urea (19ad′)

Compound **19ad′** was prepared **10** [freshly prepared from **5** (1.683 g, 5.4 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18ad′** (0.75 g, 3 mmol). Yield, 0.850 g (55%); mp 86–88 °C; ^{1}H NMR (DMSO-d_6) δ 1.37 (2H, s, CH_2), 1.49 (4H, s, 2 × CH_2), 2.42 (4H, s, 2 × CH_2), 2.63 (2H, s, CH_2), 3.69 (8H, s, 4 × CH_2), 4.00 (2H, s, CH_2), 6.50–6.52 (1H, m, ArH), 6.69–6.71 (2H, m, 2 × ArH), 6.87–6.88 (1H, m, ArH), 7.12–7.18 (2H, m, 2 × ArH), 7.26–7.28 (2H, m, 2 × ArH), 8.35 and 8.54 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{32}Cl_2N_4O_2{\cdot}5H_2O$: C, H, N.

5.4.5. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-(2-morpholinoethoxy)phenyl)urea (19ae′)

Compound **19ae′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18ae′** (1.55 g, 6 mmol). Yield, 0.948 g (31%); mp 168–169 °C; ^{1}H NMR (DMSO-d_6) δ 2.45–2.48 (4H, m, 2 × CH_2), 2.68 (2H, t, J = 6.0 Hz, CH_2), 3.58 (4H, t, J = 4.8 Hz, 2 × CH_2), 3.66–3.72 (8H, m, 4 × CH_2), 4.04 (2H, t, J = 6.0 Hz, CH_2), 6.51–6.54 (1H, m, ArH), 6.70 (2H, d, J = 9.2 Hz, 2 × ArH), 6.87 (1H, d, J = 8.0 Hz, ArH), 7.13 (1H, t, J = 8.0 Hz, ArH), 7.19 (1H, s, ArH), 7.27 (2H, d, J = 9.2 Hz, 2 × ArH), 8.31 and 8.50 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{23}H_{30}Cl_2N_4O_3{\cdot}HCl$: C, H, N.

5.4.6. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-4-(3-(2-(dimethylamino)ethoxy)phenyl)urea hydrochloride (19ba′)

Compound **19ba′** was prepared **10** [freshly prepared from **5** (1.683 g, 5.4 mmol) and triphosgene (0.623 g, 4.2 mmol)] and **18ba′** (0.65 g, 3 mmol). Yield, 0.864 g (60%); mp 203–205 °C; ^{1}H NMR (DMSO-d_6) δ 2.83 (6H, s, 2 × NMe), 3.46–3.47 (2H, m, CH_2), 3.69 (8H, s, 4 × CH_2), 4.30 (2H, t, J = 4.9 Hz, CH_2), 6.72 (2H, d, J = 8.3 Hz, 2 × ArH), 6.92 (2H, d, J = 8.8 Hz, 2 × ArH), 7.28 (2H, d, J = 8.5 Hz, 2 × ArH), 7.38 (2H, d, J = 8.8 Hz, 2 × ArH), 8.84 and 9.01 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{21}H_{28}Cl_2N_4O_2{\cdot}HCl{\cdot}H_2O$: C, H, N.

5.4.7. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-4-(3-(3-(dimethylamino)propoxy)phenyl)urea hydrochloride (19bb′)

Compound **19bb′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18bb′** (1.384 g, 6 mmol). Yield, 1.70 g (58%); mp 178–180 °C; ^{1}H NMR (DMSO-d_6) δ 2.07–2.14 (2H, m, CH_2), 2.78 (6H, s, 2 × NMe), 3.17–3.22 (2H, m, CH_2), 3.65–3.72 (8H, m, 4 × CH_2), 3.99 (2H, t, J = 6.0 Hz, CH_2), 6.69 (2H, d, J = 8.9 Hz, 2 × ArH), 6.86 (2H, d, J = 9.0 Hz, 2 × ArH), 7.26 (2H, d, J = 8.8 Hz, 2 × ArH), 7.35 (2H, d, J = 9.0 Hz, 2 × ArH), 8.62 and 8.64 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{22}H_{30}Cl_2N_4O_2{\cdot}HCl{\cdot}H_2O$: C, H, N.

5.4.8. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-4-(3-(2-(pyrrolidin-1-yl)ethoxy)phenyl)urea (19bc′)

Compound **19bc′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18bc′** (1.456 g, 6 mmol). Yield, 1.56 g (52%); mp 205–207 °C; ^{1}H NMR (DMSO-d_6) δ 1.87–1.90 (2H, m, CH_2), 2.00 (2H, br s, CH_2), 3.07–3.10 (2H, m, CH_2), 3.53–3.58 (4H, m, 2 × CH_2), 3.65–3.72 (8H, m, 4 × CH_2), 4.28 (2H, t, J = 4.9 Hz, CH_2), 6.69 (2H, d, J = 8.9 Hz, 2 × ArH), 6.93 (2H, d, J = 8.9 Hz, 2 × ArH), 7.27 (2H, d, J = 8.8 Hz, 2 × ArH), 7.38 (2H, d, J = 8.8 Hz, 2 × ArH), 8.70 and 8.87 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{23}H_{30}Cl_2N_4O_2{\cdot}1.1HCl$: C, H, N.

5.4.9. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-4-(3-(2-(piperidin-1-yl)ethoxy)phenyl)urea (19bd′)

Compound **19bd′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18bd′** (1.54 g, 6 mmol). Yield, 1.73 g (56%); mp 211–212 °C; ^{1}H NMR (DMSO-d_6) δ 1.37–1.39 (1H, m, CH), 1.67–1.70 (1H, m, CH), 1.79 (4H, br s, 2 × CH_2), 2.98 (2H, br s, CH_2), 3.43–3.50 (2H, m, 2 × CH_2), 3.67–3.72 (8H, m, 4 × CH_2), 4.34 (2H, t, J = 4.9 Hz, CH_2), 6.69 (2H, d, J = 8.9 Hz, 2 × ArH), 6.92 (2H, d, J = 8.9 Hz, 2 × ArH), 7.27 (2H, d, J = 8.9 Hz, 2 × ArH), 7.38 (2H, d, J = 8.9 Hz, 2 × ArH), 8.72 and 8.89 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{24}H_{32}Cl_2N_4O_2{\cdot}HCl$: C, H, N.

5.4.10. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-4-(3-(2-morpholinoethoxy)phenyl)urea (19be′)

Compound **19be′** was prepared **10** [freshly prepared from **5** (3.366 g, 10.8 mmol) and triphosgene (1.246 g, 4.2 mmol)] and **18be′** (1.55 g, 6 mmol). Yield, 1.33 g (43%); mp 210–212 °C; ^{1}H NMR (DMSO-d_6) δ 3.20 (2H, br s, CH_2), 3.47–3.52 (4H, m, 2 × CH_2), 3.65–3.71 (8H, m, 4 × CH_2), 3.78–3.84 (2H, m, CH_2), 3.95–3.98 (2H, m, CH_2), 4.36 (2H, t, J = 4.8 Hz, CH_2), 6.69 (2H, d, J = 9.0 Hz, 2 × ArH), 6.93 (2H, d, J = 8.9 Hz, 2 × ArH), 7.27 (2H, d, J = 8.9 Hz, 2 × ArH), 7.38 (2H, d, J = 9.0 Hz, 2 × ArH), 8.67 and 8.84 (each 1H, br s, exchangeable, 2 × NH). Anal. Calcd for $C_{23}H_{30}Cl_2N_4O_3{\cdot}HCl$: C, H, N.

5.5. Cytotoxicity assays

The in vitro cytotoxicity of the newly synthesized *N*-mustard derivatives 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[28] and human solid tumor cells (i.e., breast carcinoma MX-1 and colon carcinoma HCT-116) by the SRB assay[29] in a 72 h incubation using a microplate spectrophotometer as previously described.[30] After the addition of phena-

zine 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 PC3, human lung adenocarcinoma (CL 1–0 and CL 1–5) and human breast adenocarcinoma MCF-7 were determined by the Alamar blue assay[31] in a 72 h incubation using a microplate spectrophotometer as previously described. After the addition of Alamar blue solution, it was incubated at 37 °C for 6 h. Absorbance at 570 and 600 nm was detected on a microplate reader. IC_{50} values were determined from dose-effect relationship at six or seven concentrations of each drug using the CompuSyn software by Chou and Martin[32] based on the median-effect principle and plot.[33,34] Ranges given for taxol, vinblastine and cisplatin were mean ± SE (n = 4).

5.6. In vivo studies

Athymic nude mice bearing the nu/nu gene were obtained from NCI, Frederick, MD and used for all human tumor xenografts. Male nude mice 6 weeks or older weighing 20–24 g or more were used. Compound **9aa′** was administered via the tail vein for iv injection as previously described.[30] Tumor volume was assessed by measuring length × width × height (or width) by using a 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. For tumor-bearing nude mice during the course of the experiment, the body-weight referred to total body weight minus the weight of the tumor assuming 1 mm^3 = 1 mg. All animal studies were conducted in accordance with the guidelines for the National Institute of Health Guide for the Care and Use of Animals and the protocol approved by the Institutional Animal Care and Use Committee.

5.7. Alkaline agarose gel shift assay

Formation of DNA cross-linking was analyzed by alkaline agarose gel electrophoresis. In brief, purified pEGFP-N1 plasmid DNA (1500 ng) was mixed with various concentrations (1–20 μM) of **9aa′**, **9ad′**, **19bd′** and **19bb′** in 40 μL binding buffer (3 mM sodium chloride/1 mM sodium phosphate, pH 7.4, and 1 mM EDTA). The reaction mixture was incubated at 37 °C for 2 h. At the end of the reaction, the plasmid DNA was linearized by digestion with BamHI and followed by precipitation with ethanol. The DNA pellets were dissolved and denatured in alkaline buffer (0.5 N NaOH–10 mM EDTA). An aliquot of 20 μL of DNA solution(1 μg) was mixed with 4 μL of 6% alkaline loading dye and then electrophoretically resolved on a 0.8% alkaline agarose gel with NaOH–EDTA buffer at 4 °C. The electrophoresis was carried out at 18 V for 22 h. After staining the gels with an ethidium bromide solution, the DNA was then visualized under UV light.

5.8. Cell cycle inhibition

The effects of **9aa′** on cell cycle distribution were analyzed with a flow cytometer as previously described.[35] Briefly, human non-small cell lung carcinoma H1299 cells were treated with **9aa′** at 2.5, 5, and 10 mM for 24 h. The attached cells were then trypsinized, washed with phosphate buffer saline (PBS), and fixed with ice-cold 70% ethanol for 30 min. The cells were stained with 4 μg/ml propidium iodide (PI) in PBS containing 1% Triton X-100 and 0.1 mg/ml RNase A. The stained cells were then analyzed using the FACS SCAN flow cytometer (Becton Dickinson, San Joes, CA, USA). The percentage of the cells in each cell cycle phase was determined using the ModFit LT 2.0 software based on the DNA histograms.

5.9. In vivo pharmacokinetic study/drug administration and sampling

All procedures of the present study were in accordance with the IACUC guidelines. Prior approval of the Institutional Animal Ethics Committee (IAEC) was obtained before initiation of the study. A single intravenous dose of **9aa′** was administered via an indwelling catheter in jugular vein to two male Sprague Dawley rats at a dose level of 1.0 mg/kg. The formulation was prepared as a solution in 5.0% w/v DMSO with 10% w/v Cremophor in double distilled water. Blood samples were collected at 0.083, 0.166, 0.333, 0.666, 1, 2, 4, 6, and 8 h after fasted from twelve hours prior to dosing. Serial blood samples (~200 μL/each) were collected from all animals via the carotid artery cannulations. Samples were placed into tubes containing 10 μL of 350 IU/mL heparin solution. Plasma was harvested from the blood by centrifugation at 4000 rpm for 10 min at 4 ± 2 °C. All samples were stored at approximately −80 °C until bioanalysis.

5.10. Bioanalytical method

Concentration levels of **9aa′** in rat plasma samples were determined by partially validated LC–MS/MS method. The calibration curve was linear from 2.5 to 300 ng/mL for **9aa′**. The coefficient of determination (r^2) was greater than 0.999. The plasma samples were quenched by adding 90 μL of acetonitrile (0.1% formic acid) containing the internal standard (BO-1441, 100 ng/mL) to 30 μL of sample. The plasma samples were vortex-mixed briefly at high speed, kept on ice for 10 min, and then centrifuged at 12,000 rpm for 5 min. Approximately 100 μL of the supernatant of each tube was transferred to an amber clean autosampler vial with insert for analysis. A 5 μL of the aliquot solution was subsequently injected into the LC–MS/MS system.

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Supplementary data

Supplementary data (detailed experimental procedures for the synthesis of intermediate compounds **15a,b**, **17ae′–17be′**, and **18aa′–18be′** along with their spectroscopic data, and elemental analysis data of all unknown compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.005.

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