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Potent DNA-directed alkylating agents: Synthesis and biological activity of phenyl *N*-mustard–quinoline conjugates having a urea or hydrazinecarboxamide linker

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ABSTRACT

A series of *N*-mustard–quinoline conjugates bearing a urea or hydrazinecarboxamide linker was synthesized for antitumor evaluation. The in vitro cytotoxicity studies revealed that compounds with hydrazinecarboxamide linkers were generally more cytotoxic than the corresponding urea counterparts in inhibiting human lymphoblastic leukemia and various solid tumor cell growths in culture. The therapeutic efficacy against human tumor xenografts in animal model was studied. It was shown that complete tumor remission in nude mice bearing human breast carcinoma MX-1 xenograft by **17a, i** and **18c, d** was achieved. In the present study, it was revealed that both linkers are able to lower the chemically reactive *N*-mustard pharmacophore and thus the newly synthesized conjugates possess a long half-life in rat plasma. Moreover, the new *N*-mustard derivatives are able to induce DNA cross-linking either by modified comet assay or by alkaline agarose gel shift assay.

1. Introduction

DNA alkylating agents have been widely used in chemotherapy.[1–3] However, the progress of developing new alkylating agents is sluggish since these derivatives have several drawbacks including a lack of drug-specific affinity to tumor cells, a high chemical reactivity and induced bone marrow toxicity.[4–6] Recently, there is renewed interest in the discovery of new alkylating agents for cancer chemotherapy via the designing of DNA-directed alkylating agents or prodrug to overcome the general drawbacks of alkylating agents.

DNA-directed alkylating agents are generally synthesized by linking alkylating pharmacophores (such as *N*-mustard residue) to DNA-affinic molecules (such as DNA intercalating agents or DNA minor groove binder).[7–10] It has been demonstrated that these agents have higher cytotoxicity and better therapeutic efficacy than the corresponding untargeted alkylating agents. Studies on the structure–activity relationships of these conjugates suggest that selection of DNA-affinic molecule (carrier), *N*-mustard residue (alkyl or phenyl *N*-mustard) and the spacer (type and length) greatly affect their antitumor activity. For example, Tallimustine (**1**, FCE 24517, Chart 1)[11] was selected as an anticancer drug candidate of this new class of cytotoxic compounds. It was evaluated in Phase I and II clinical trials and expressed a promising anticancer activity both in vivo and in vitro.[11]

As for designing prodrugs, Springer et al. and other laboratories synthesized various *N*-mustard prodrugs by masking phenyl *N*-mustards with glutamic acid or tyramine via a urea (**2**, X = NH,[12] **3**,[13] and **4**,[14] Chart 1), carbamate (**2**, X = O;[12] and **5**,[15]), and carboxamide [**6** (CMDA)[16,17] and **7** (CJS 1050)[18]] spacer for antibody-directed enzyme prodrug therapy (ADEPT),[19] gene-directed enzyme prodrug therapy (GDEPT)[20] or melanocyte-directed enzyme prodrug therapy (MDEPT).[13–15] These studies suggest that urea, carbamate, and carboxamide linker are able to lower the reactivity of the reactive *N*-mustard pharmacophore.

In our research on developing DNA-directed alkylating agents, we previously exemplified that alkyl *N*-mustard residue linked to the anilino ring or/and acridine chromophore of 9-anilinoacridines (alkyl *N*-mustard–9-anilinoacridine conjugates) or acridine via a methylene or alkoxy ($O–C_{1–4}$) linker possesses significant cytotoxicity in inhibiting various human leukemia and solid tumor cell growth in vitro as well as potent therapeutic effects against human tumor xenografts in animal model.[21–24] We have also shown that the 9-anilinoacridine conjugates were more cytotoxic than the corresponding acridine derivatives. Moreover, we found that the length of the spacer also influences their potency. Among these

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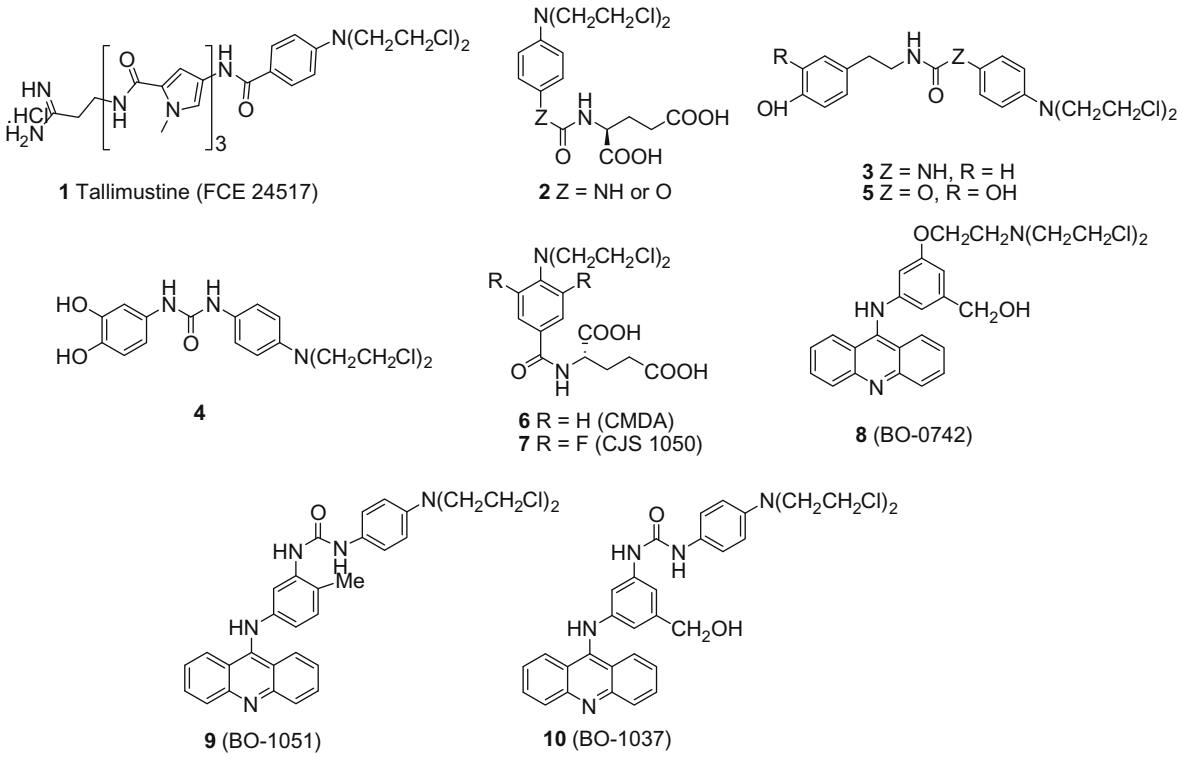


Chart 1. Chemical structure of *N*-mustard derivatives.

conjugates, BO-0742 (**8**, Chart 1) was capable of achieving complete tumor remission against human breast MX-1 xenograft or significant suppression of human T-cell acute lymphoblastic leukemia CCRF-CEM, colon carcinoma HCT-116, and ovarian adenocarcinoma SK-OV-3 tumor xenografts in animal models. However, as BO-0742 has a narrow therapeutic window and is chemically unstable with a short half-life in mice, it has low bioavailability. To overcome the poor pharmacokinetics of BO-0742, we have recently synthesized a series of phenyl *N*-mustard–9-anilinoacridine conjugates that have urea and carbamates linkers.[25,26] These studies revealed that the conjugates were more chemically stable, exhibited potent therapeutic efficacy against human xenografts in vivo, and are also able to induce DNA interstrand cross-linking in tumor cells. Among these agents, we found that BO-1051 (**9**) had a longer half-life in rat plasma and a broader spectrum of antitumor activity than BO-0742 had.

To continue our research on developing new potential DNA-directed alkylating agents, quinolines were chosen as DNA-affinic molecule to replace 9-anilinoacridines, as they are DNA minor groove binders.[27] In these new *N*-mustard–quinoline conjugates, urea or hydrazinecarboxamide were used as stabilizing spacers. The newly synthesized conjugates would allow us to understand whether using quinolines as a carrier has any advantage over the usage of 9-anilinoacridines, as well as the role of the urea or hydrazinecarboxamide spacer in affecting their antitumor effects and chemical stability. The new target derivatives were subjected to antitumor evaluation against a variety of human tumor cell growth in vitro, therapeutic efficacy in vivo, and their capability of DNA interstrand cross-linking. The results of these studies are presented in this paper.

2. Result and discussion

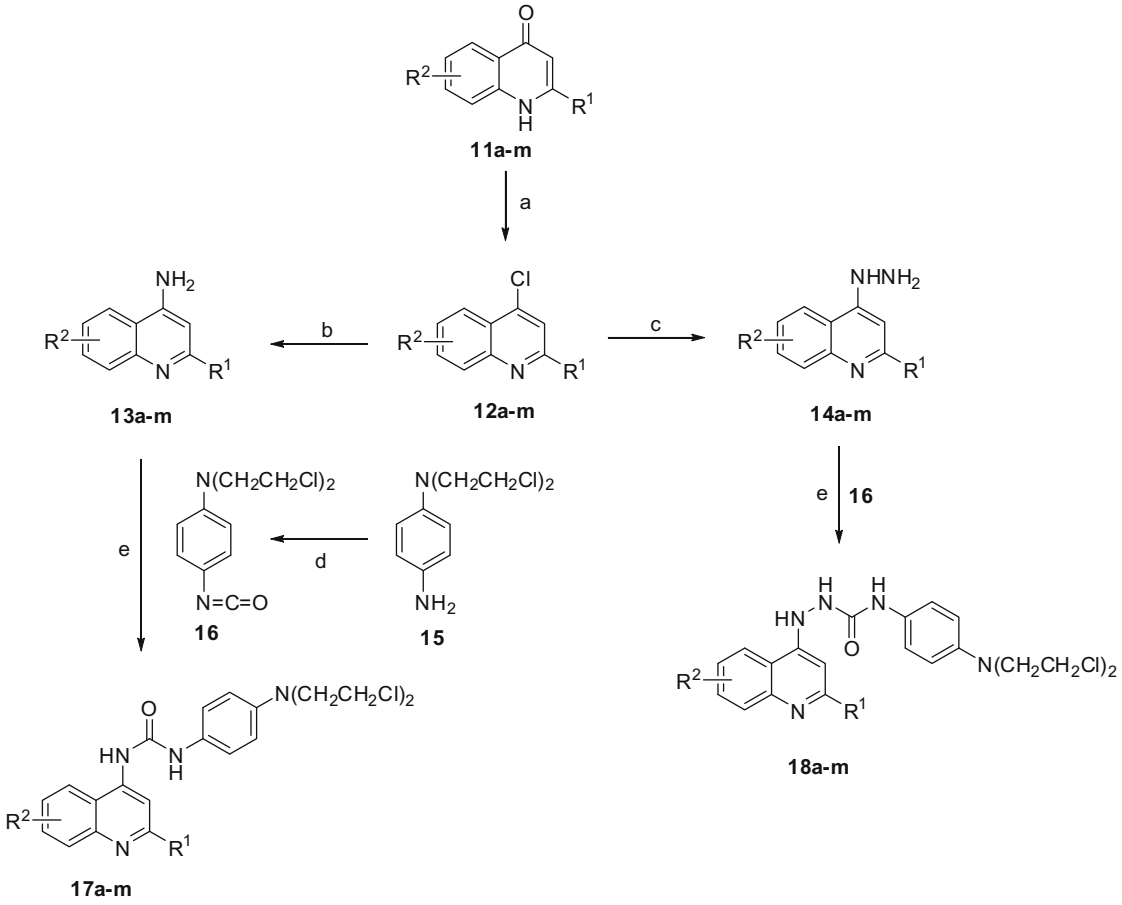
2.1. Chemistry

The general synthetic route of the newly *N*-mustard–quinoline conjugates was summarized in Scheme 1. 4-Chloro-2-methylquinoline (**12a**) and 4-amino-2-methylquinoline (**13a**) were commercially available. The substituted 4-chloroquinolines (**12b–m**) were synthesized from the corresponding known 4-quinolinones (**11b–m**)[28–30] via chlorination[31] using phosphorus oxychloride ($POCl_3$). Treatment of **12b–m** with ammonia in phenol at 180 °C by following the literature procedure[32] gave 4-aminoquinolines (**13b–m**). The 4-hydrazinoquinoline derivatives (**14a–d, f–m**) used for preparing **18a–d, f–m** were obtained by the treatment of the appropriate 4-chloroquinolines (**12a–d, f–m**) with hydrazine hydrate as previously described.[33] Condensation of 4-aminoquinolines (**13a–m**) and 4-hydrazinoquinolines (**14a–d, f–m**) with 4-[*N*,*N*-bis(2-chloroethyl)amino]phenylisocyanate (**16**),[34] [freshly prepared by reaction of *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**15**),[15] with triphosgene] in anhydrous DMF in the presence of triethylamine (TEA) afforded the desired *N*-mustard–quinoline conjugates bearing a urea (**17a–m**) or hydrazincarboxamide (**18a–d, f–m**) linker, respectively, with fair to good yield (Table 1).

2.2. Biological results

2.2.1. In vitro cytotoxicity

The antiproliferative activities of the newly synthesized *N*-mustard–quinoline conjugates bearing a urea or hydrazinecarboxamide linker (**17a–m, 18a–d**, and **18f–m**, respectively) against human lymphoblastic leukemia (CCRF-CEM), cell growth in vitro are summarized in Table 2. The structure–activity relationship (SAR) studies clearly show that the conjugates with hydrazinecarboxamide linkers (**18a–d**, **f–m**) are generally more cytotoxic than the corresponding derivatives bearing a urea linker (**17a–m**). The 2-Me substituted conjugates in both series of compounds (R^1 = Me, **17a–e**, **18a–d**) are generally more cytotoxic than the corresponding 2-Ph, 2-(3-MeOPh) or 2-(2-FPh) substituted counter parts. In the series of 2-Me substituted compounds bearing a urea linker, compound **17d** with the electron-donating *N*,*N*-dimethyl function at C-6 is slightly more cytotoxic than the C-6 unsubstitut-



Scheme 1. Synthesis of phenyl *N*-mustard–quinoline conjugates having a urea and hydrazinecarboxamide linker. Reagents and conditions: (a) $POCl_3$, reflux; (b) NH_3/phenol, 180 °C; (c) NH_2NH_2/ethanol, reflux; or phenol/NH_2NH_2, 140 °C; (d) triphosgene/$CHCl_3$/Et_3N, 0 °C; (e) Et_3N, DMF, room temperature.

ed **17a**. However, the cytotoxicity of this conjugate is decreased by adding a methylenedioxy, methoxy or pyrrolidinyl function at C-6,7 or C-6 (i.e., **17b**, **17c**, or **17e**, respectively). In the series of 2-phenyl substituted derivatives, the C-6 unsubstituted compound **17f** is slightly more cytotoxic than **17g** (C-6,7-OCH_2O–). While compounds with 3′-MeOPh function at C-2 (**17h–k**), **17i** (C-6 OMe) have better antiproliferative activity than that of **17h** (C-6 Cl) and **17j** (C-6,7-OCH_2O–) counterparts. It is interested to note that the introduction of a pyrrolidinyl substituent at C-6 greatly decreases the cytotoxicity (**17e**, **17k**, and **17m**). In the series of compounds with a hydrazinecarboxamide linker, it is surprise to find that among the C-2 Me substituted derivatives (**18a–d**), Compound **18c**, which bears a methylenedioxy substituent at C-6,7, is the most cytotoxic in inhibiting CCRF-CEM cell growth in vitro with an IC_{50} value of 0.042 μM. A similar observation is found in compounds bearing a C-2-Ph (**18f, g**), C-2-3′-MeOPh derivatives (**18h–k**), or C-2-2′-F-Ph conjugates (**18l**, **m**). In contrast with urea derivatives (**17k, m**), the cytotoxicity of hydrazinecarboxamide conjugates is greatly increased by the introduction of the C-6 pyrrolidinyl substituted at C-6 (**18k, m**).

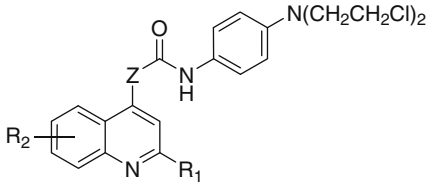
One of the important goals of new drug development is the discovery of agents that are not multidrug resistant to distinct drugs. We used CCRF-CEM/Taxol and CCRF-CEM/VBL, which are subcell lines of CCRF-CEM cells that are 330-fold resistant to taxol and 680-fold resistant to vinblastine, respectively, in comparison with the IC_{50} of the parent cell line, to study whether the newly synthesized conjugates exhibit multidrug resistance towards taxol or vinblastine (Table 2). The results revealed that conjugates with a urea or hydrazinecarboxamide linker have little or no cross-resistance to either taxol or vinblastine with the exception of compound **18c**, which has a certain degree of cross-resistance to both anticancer agents.

The newly synthesized compounds were further evaluated for their cytotoxicity against various human solid tumor cell growths in vitro (Table 3). Similarly, the urea conjugates are generally more cytotoxic than the corresponding hydrazinecarboxamide counters in inhibiting MX-1 and HCT-116, with the exception of **18h** and **18i**, which were less active than the corresponding **17h** and **17i**, respectively. Similarly, compounds with a pyrrolidinyl substituent at C-6 in urea conjugates (**17e**, **17l**, and **17m**) were found to be much less active against the same tested tumor cell lines. To further explore the antiproliferative activity of the new *N*-mustard–quinoline conjugates, the selected compounds were studied their cytotoxicity in inhibiting other human solid tumors such as human non-small cell lung cancer (H1299), lung adenocarcinoma (CL 1-0 and CL 1-5), prostate cancer (PC-3) and resistant breast cancer (MCF-7) cell growth in vitro. As shown in Table 3, one can see that these conjugates possess good to moderate cytotoxic effects against the growth of these cell lines in vitro.

2.2.2. In vivo therapeutic efficacy

The newly synthesized conjugates were selected for evaluation of their antitumor effects against human tumor xenografts in animal models based on their in vitro cytotoxicity, toxicity to the host and solubility. The administration of compounds was carried out at the maximal tolerable dose via either intravenous infusion (iv infusion) or intravenous injection (iv injection) as shown in Table 4. The results of the in vivo therapeutic evaluation of *N*-mustard–

Table 1
Yield and physical data table of the compounds **17a–m**, **18a–d**, **f–m**



Z = NH or NH-NH

Compd	R^1	R^2	Yield (%)	Mp (°C)	Chemical formula	Analysis
17a	Me	H	85	174–175	$C_{21}H_{22}Cl_2N_4O$	C, H, N
17b	Me	6-MeO	77	251–252	$C_{22}H_{24}Cl_2N_4O_2$	C, H, N
17c	Me	6,7-(OCH_2O)	58	271–272	$C_{22}H_{22}Cl_2N_4O_3$	C, H, N
17d	Me	6-NMe_2	65	267–268	$C_{23}H_{27}Cl_2N_5O$	C, H, N
17e	Me	6-C_4H_8N	63	255–256	$C_{25}H_{29}Cl_2N_5O$	C, H, N
17f	C_6H_5	H	73	194–195	$C_{26}H_{24}Cl_2N_4O$	C, H, N
17g	C_6H_5	6,7-(OCH_2O)	65	191–192	$C_{27}H_{24}Cl_2N_4O_3$	C, H, N
17h	3-MeO–C_6H_4	6-Cl	78	>300	$C_{27}H_{25}Cl_3N_4O_2$	C, H, N
17i	3-MeO–C_6H_4	6-MeO	74	255–256	$C_{28}H_{28}Cl_2N_4O_3$	C, H, N
17j	3-MeO–C_6H_4	6,7-(OCH_2O)	69	202–203	$C_{28}H_{26}Cl_2N_4O_4$	C, H, N
17k	3-MeO–C_6H_4	6-C_4H_8N	78	166–167	$C_{31}H_{33}Cl_2N_5O_2$	C, H, N
17l	2-F–C_6H_4	6,7-(OCH_2O)	80	188–189	$C_{27}H_{23}Cl_2FN_4O_3$	C, H, N
17m	2-F–C_6H_4	6-C_4H_8N	70	199–200	$C_{30}H_{30}Cl_2FN_5O$	C, H, N
18a	Me	H	94	263–264	$C_{21}H_{23}Cl_2N_5O$	C, H, N
18b	Me	6-MeO	60	255–256	$C_{22}H_{25}Cl_2N_5O_2$	C, H, N
18c	Me	6,7-(OCH_2O)	80	253–254	$C_{22}H_{23}Cl_2N_5O_3$	C, H, N
18d	Me	6-NMe_2	68	260–261	$C_{23}H_{28}Cl_2N_6O$	C, H, N
18f	C_6H_5	H	60	235–236	$C_{26}H_{25}Cl_2N_5O$	C, H, N
18g	C_6H_5	6,7-(OCH_2O)	64	289–291	$C_{27}H_{25}Cl_2N_5O_3$	C, H, N
18h	3-MeO–C_6H_4	6-Cl	61	237–238	$C_{27}H_{26}Cl_3N_5O_2$	C, H, N
18i	3-MeO–C_6H_4	6-MeO	71	215–216	$C_{28}H_{29}Cl_2N_5O_3$	C, H, N
18j	3-MeO–C_6H_4	6,7-(OCH_2O)	73	279–280	$C_{28}H_{27}Cl_2N_5O_4$	C, H, N
18k	3-MeO–C_6H_4	6-C_4H_8N	68	190–191	$C_{31}H_{34}Cl_2N_6O_2$	C, H, N
18l	2-F–C_6H_4	6,7-(OCH_2O)	78	211–212	$C_{27}H_{24}Cl_2FN_5O_3$	C, H, N
18m	2-F–C_6H_4	6-C_4H_8N	62	270–271	$C_{30}H_{31}Cl_2FN_6O$	C, H, N

quinoline conjugates are summarized in Table 4. It shows that the therapeutic effect of *N*-mustard–quinoline conjugate **18a** on human breast carcinoma MX-1 xenograft subcultivated in nude mice in a dose-dependent manner. At the dose of 10 mg/kg (Q2D×7, 2 h iv infusion), the tumors were significantly suppressed, but relapse occurred on D30. However, at the dose of 15 mg/kg (Q2D×7, 2 h iv infusion), tumor remission was observed (4/4 remission on D26, D26, D32, and D38), with only one mouse showing tumor relapse on D42. Under this dose, about 8% average body-weight loss was found indicating that **18a** caused litter toxicity in the host (see Supplementary data 1AB). In a similar experiment, we found that **18c** and **18d** achieved complete tumor remission (CR) (for **18c**: on D19, D21, D21, and D23; for **18d**: D17, D19, D19, and D21) at the maximal tolerable dose of 40, 40 mg/kg (Q2D×6, iv injection), respectively (Fig. 1A and B). The mice showed a 15% body-weight loss on day 23 indicating that the methylenedioxy or dimethylamino function at C-6 did not affect the potency of these two agents.

As mentioned previously, *N*-mustard–quinoline conjugates with a urea linker are generally less cytotoxic than the corresponding derivatives bearing a hydrazinecarboxamide spacer. We studied the antitumor activity of the urea conjugates, **17a** and **17i**. The results are shown in Table 4 and Figure 2. It shows that CR was also achieved by treating these two agents at the higher tolerable dose of 150 and 100 mg/kg (Q2D×5, iv injection, $n = 5$), respectively, in nude mice bearing MX-1 xenograft and maintained no relapse over 70 days. The results showed that while **17i** was more toxic than **17a**, the body-weight of the mice recovered after discontinuation of the treatment. The studies demonstrated that the maximal effective doses for the urea conjugates are higher than that for the hydrazinecarboxamide derivatives. Furthermore, we compared the antitumor effects of **17a** with *N*-mustard–AHMA conjugate with the urea linker BO-1037 (**10**, Chart 1) in MX-1 xenograft model (Fig. 3). The results clearly show that both conjugates are able to induce CR at doses of 150 and 100 mg/kg, respectively, via 2 h iv infusion. Although one out of four mice died due to the toxicity of **10** during treatment, the other treated mice recovered soon after the cessation of drug administration. The experiments suggest that the *N*-mustard–quinoline conjugates may be less toxic to the host than *N*-mustard–9-anilinoacridine conjugates.

We further investigated the therapeutic efficacy of **17a** against human multidrug-resistant breast cancer MCF-7/Adr xenograft in nude mice and compared it with that of gemcitabine, cyclophosphamide and taxol. As shown in Figure 4A and B, it can be clearly seen that while **17a** is as potent as gemcitabine, it is also more effective than either cyclophosphamide or taxol and is relatively less toxic among the compounds tested.

Conjugates **18c** and **18d** were also selected for evaluating their therapeutic efficacy against human colon HCT-116 xenograft in nude mice. Table 4 (Supplementary data 2) shows that there were 60% and 78% tumor suppression by **18c** and **18d** at the maximum tolerable dose of 30 and 50 mg/kg, respectively, with acceptable toxicity (about 12–13% body-weight loss).

The above studies demonstrate that the *N*-mustard–quinoline conjugates with hydrazinecarboxamide linkers are generally more potent than the corresponding derivatives bearing urea linkers regarding the dose used. However, the later conjugates possess lower toxicity to the host. The current studies also suggest that the newly synthesized compounds may have superior antitumor efficacy against multidrug-resistant tumors since these conjugates, especially conjugates bearing urea linkers, have little or no multidrug resistance as previously described.

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) cell growth in vitro

Compd	R^1	R^2	IC_{50}[a] (μM)		
			CCRF-CEM	CCRF-CEM/Taxol[b]	CCRF-CEM/VBL[b]
17a	Me	H	0.193 ± 0.004	0.429 ± 0.009 [2.2×][c]	0.664 ± 0.014 [3.4×]
18a	Me	H	0.118 ± 0.053	1.83 ± 0.8 [15.5×]	0.85 ± 0.05 [7.2×]
17b	Me	6-MeO	0.309 ± 0.057	0.313 ± 0.006 [1.0×]	0.458 ± 0.003 [1.5×]
18b	Me	6-MeO	0.196 ± 0.019	1.535 ± 0.385 [7.8×]	2.015 ± 0.181 [10.3×]
17c	Me	6,7-(OCH_2O)	0.279 ± 0.025	0.605 ± 0.008 [2.2×]	0.652 ± 0.027 [2.3×]
18c	Me	6,7-(OCH_2O)	0.042 ± 0.02	2.26 ± 0.65 [53.8×]	1.725 ± 0.39 [41.1×]
17d	Me	6-NMe_2	0.107 ± 0.023	0.308 ± 0.001 [2.9×]	0.242 ± 0.01 [2.3×]
18d	Me	6-NMe_2	0.058 ± 0.026	4.193 ± 0.651 [72.3×]	6.95 ± 5.86 [120×]
17e	Me	6-C_4H_8N	9.02 ± 0.011	16.873 ± 0.792 [1.87×]	12.931 ± 0.018 [1.43×]
17f	C_6H_5	H	1.686 ± 0.024	1.462 ± 0.115 [0.87×]	1.664 ± 0.083 [0.99×]
18f	C_6H_5	H	0.578 ± 0.002	0.908 ± 0.0025 [1.57×]	1.217 ± 0.012 [2.11×]
17g	C_6H_5	6,7-(OCH_2O)	2.252 ± 0.222	2.229 ± 0.006 [0.99×]	1.837 ± 0.036 [0.82×]
18g	C_6H_5	6,7-(OCH_2O)	0.258 ± 0.044	0.574 ± 0.0021 [2.22×]	0.495 ± 0.0057 [1.92×]
17h	3-MeO–C_6H_4	6-Cl	1.88 ± 0.43	1.104 ± 0.066 [0.59×]	1.029 ± 0.02 [0.55×]
18h	3-MeO–C_6H_4	6-Cl	0.732 ± 0.22	2.253 ± 0.042 [3.08×]	2.133 ± 0.051 [2.91×]
17i	3-MeO–C_6H_4	6-MeO	0.362 ± 0.004	0.588 ± 0.001 [1.62×]	0.557 ± 0.006 [1.53×]
18i	3-MeO–C_6H_4	6-MeO	0.552 ± 0.064	2.154 ± 0.193 [3.90×]	1.623 ± 0.088 [2.94×]
17j	3-MeO–C_6H_4	6,7-(OCH_2O)	2.816 ± 0.788	1.961 ± 0.055 [0.70×]	2.224 ± 0.042 [0.79×]
18j	3-MeO–C_6H_4	6,7-(OCH_2O)	0.209 ± 0.045	0.734 ± 0.013 [3.51×]	1.134 ± 0.02 [5.43×]
17k	3-MeO–C_6H_4	6-C_4H_8N	102.15 ± 13.79	2.227 ± 0.013 [0.02×]	774.46 ± 8.625 [7.58×]
18k	3-MeO–C_6H_4	6-C_4H_8N	0.354 ± 0.031	0.568 ± 0.006 [1.60×]	0.833 ± 0.044 [2.35×]
17l	2-F–C_6H_4	6,7-(OCH_2O)	3.087 ± 0.508	2.607 ± 0.011 [0.84×]	2.348 ± 0.019 [0.76×]
18l	2-F–C_6H_4	6,7-(OCH_2O)	1.423 ± 0.270	2.979 ± 0.024 [2.09×]	3.174 ± 0.205 [2.23×]
17m	2-F–C_6H_4	6-C_4H_8N	7.647 ± 0.978	39.896 ± 2.831 [5.22×]	12.423 ± 0.588 [1.62×]
18m	2-F–C_6H_4	6-C_4H_8N	0.152 ± 0.02	0.261 ± 0.030 [1.72×]	0.312 ± 0.057 [2.05×]
Taxol			0.0031 ± 0.0003	0.429 ± 0.042 [330×]	1.274 ± 0.052 [980×]
Vinblastine			0.00073 ± 0.0009	0.078 ± 0.011 [106.2×]	0.496 ± 0.121 [679.5×]

[a] Cell growth inhibition was measured by the XTT assay[38] for leukemic cells and the SRB assay[39] for solid tumor cells after 72-h incubation using a microplate spectrophotometer as described previously.[40] 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[42] based on the median-effect principle and plot.[43,44] 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.

2.2.3. Chemical stability in rat plasma

Our previous report demonstrated that urea or carbamate linker in phenyl *N*-mustard–9-anilinoacridine conjugates are capable of lowing the reactivity of the reactive *N*-mustard pharmacophore.[25,26] Therefore, these conjugates are stable and have a long half-life in rat plasma. In the present study, we investigated the chemical stability of **17a** in rat plasma. The result showed that **17a** is also chemically stable in rat plasma with a long half-life ($t_{1/2}$ = 16.48±3.68 h, $n = 3$) indicating that the newly prepared *N*-mustards are chemically and metabolically stable derivatives.

2.2.4. DNA cross-linking study by alkaline agarose gel shift assay

The alkaline gel shift assay was performed to assess DNA cross-linking activity of compounds **17a**, **18d** and **18f**, and **18i** for their

Table 3
Cytotoxicity of new *N*-mustards against human solid tumors (MX-1, HCT-116, H1299, CL 1-0, CL 1-5, PC-3 and MCF-7) cell growth in vitro

Compd	IC_{50} (μM)						
	MX-1	HCT-116	H1299	CL 1-0	CL 1-5	PC-3	MCF-7
17a	0.343 ± 0.006	1.423 ± 0.126	1.984 ± 0.074	9.024 ± 0.606	9.449 ± 1.161	9.515 ± 3.738	9.084 ± 1.169
18a	0.36 ± 0.072	0.382 ± 0.029	0.784 ± 0.056	9.441 ± 2.915	8.869 ± 2.38	5.963 ± 0.572	5.549 ± 1.357
17b	0.645 ± 0.007	0.83 ± 0.033	1.307 ± 0.074	5.583 ± 0.941	5.333 ± 0.366	3.152 ± 0.591	5.188 ± 0.791
18b	0.264 ± 0.005	0.257 ± 0.002	3.63 ± 0.114	3.547 ± 0.637	6.261 ± 0.744	7.162 ± 1.074	14.775 ± 1.617
17c	0.584 ± 0.008	0.402 ± 0.014	5.443 ± 0.377	6.229 ± 0.335	7.274 ± 0.826	5.965 ± 1.061	4.906 ± 0.423
18c	0.127 ± 0.002	0.123 ± 0.003	0.812 ± 0.136	1.115 ± 0.921	2.712 ± 0.905	2.322 ± 0.579	2.534 ± 1.071
17d	0.184 ± 0.001	0.3 ± 0.008	0.714 ± 0.089	1.727 ± 0.686	2.523 ± 1.08	2.001 ± 0.433	2.134 ± 0.739
18d	0.278 ± 0.013	0.145 ± 0.002	0.289 ± 0.112	1.832 ± 0.536	4.326 ± 0.963	1.289 ± 0.387	3.487 ± 0.444
17e	16.63 ± 3.48	13.388 ± 0.036	N.D.	N.D.	N.D.	N.D.	N.D.
17f	3.474 ± 0.083	7.792 ± 0.106	N.D.	N.D.	N.D.	N.D.	N.D.
18f	1.776 ± 0.036	1.258 ± 0.059	4.378 ± 0.261	4.071 ± 0.591	3.013 ± 0.532	10.524 ± 0.6004	6.242 ± 0.225
17g	7.412 ± 0.406	9.916 ± 0.198	N.D.	N.D.	N.D.	N.D.	N.D.
18g	1.024 ± 0.023	2.024 ± 0.126	N.D.	N.D.	N.D.	N.D.	N.D.
17h	2.246 ± 0.059	4.88 ± 0.007	N.D.	N.D.	N.D.	N.D.	N.D.
18h	14.587 ± 1.046	13.888 ± 0.223	N.D.	N.D.	N.D.	N.D.	N.D.
17i	1.832 ± 0.036	3.689 ± 0.040	5.689 ± 0.124	4.726 ± 0.302	3.818 ± 0.325	5.883 ± 0.791	3.043 ± 0.774
18i	3.459 ± 0.119	3.444 ± 0.045	5.992 ± 0.581	11.679 ± 0.822	10.96 ± 0.329	6.449 ± 0.688	15.385 ± 1.356
17j	20.624 ± 0.412	8.867 ± 0.863	N.D.	N.D.	N.D.	N.D.	N.D.
18j	0.749 ± 0.007	1.374 ± 0.063	2.054 ± 0.256	2.871 ± 0.675	4.846 ± 0.442	2.983 ± 0.215	3.347 ± 0.633
17k	264.63 ± 10.77	167.68 ± 11.95	3.812 ± 0.404	7.093 ± 1.421	9.494 ± 1.226	11.09 ± 0.199	6.927 ± 1.037
18k	1.323 ± 0.061	0.643 ± 0.012	0.945 ± .081	2.916 ± 0.515	5.865 ± 0.242	3.336 ± 0.609	5.455 ± 0.792
17l	13.076 ± 0.089	25.396 ± 2.185	N.D.	N.D.	N.D.	N.D.	N.D.
18l	42.579 ± 0.192	6.476 ± 0.521	0.491 ± 0.083	3.472 ± 0.701	2.65 ± 0.417	4.774 ± 0.811	1.819 ± 0.185
17m	114.97 ± 17.89	44.215 ± 3.101	N.D.	N.D.	N.D.	N.D.	N.D.
18m	2.264 ± 0.099	0.192 ± 0.012	0.604 ± 0.071	0.694 ± 0.15	0.511 ± 0.0625	0.954 ± 0.195	1.002 ± 0.146
Taxol	0.035 ± 0.00514	0.0013 ± 0.0005	N.D.	N.D.	N.D.	N.D.	N.D.
Vinblastine	0.0029 ± 0.0002	0.0018 ± 0.0004	N.D.	N.D.	N.D.	N.D.	N.D.
Cisplatin	N.D.	N.D.	4.948 ± 0.604	55.832 ± 3.834	23.863 ± 1.478	26.654 ± 4.194	23.113 ± 1.487

Table 4
The antitumor therapeutic effect of the representative compounds in nude mice bearing human breast carcinoma MX-1 xenografts

Compound	Dose (mg/kg)	Schedule	Effect Tumor free or tumor volume change (%)	Toxicity Body-weight loss (%)
18a[a]	10	Q2D×7 2 h iv infusion	1/4 relapsed on D30	2% loss on D20 Recover after D26
18a[a]	15	Q2D×7 2 h iv infusion	4/4 remission on D26, D26, D32, D38 1/4 relapse on D42	8% loss on D20 6% loss on D46
18c[a]	40	Q2D×6 iv injection	4/4 complete remission on D19,21, 21, 23	16% loss on D23
18c[a]	30	Q2D×6 iv injection	60% supression on D22	12% loss on D22
18d[a]	40	Q2D×6 iv injection	4/4 complete remission on D17,19, 19, 21	15% loss on D20
18d[b]	50	Q2D×6 iv injection	78% supression on D22	12.5% loss on D22
17a[a]	150	Q2D×5 iv injection	5/5 CR on D16 No relapse on D70	2% loss on D16 Recover after D18
17i[a]	100	Q2D×5 iv injection	5/5 CR on D18 No relapse on D70	13% loss on D16

[a] Human breast carcinoma MX-1 xenograft.
[b] Human colon HCT-116 xenograft.

wide range of IC_{50} values to H1299 cells (Fig. 5). This study shows that all the test compounds were able to interact with the plasmid DNA and form interstrand cross-links, which could not be separated into single strand under alkaline condition. The cross-linking ability of the tested compounds was comparable with the melphalan (positive control) which shows cross-links at lower concentrations of treatment. Test compounds show moderate cross-linking behavior at lower concentrations, but at high concentrations the DNA bands got smeared which was similar with melphalan at high concentrations of treatment. These results revealed that the newly synthesized *N*-mustard–quinoline conjugates are capable of inducing DNA cross-linking formation.

2.2.5. DNA interstrand cross-linking study (modified comet assay)

To understand whether the newly synthesized derivatives are able to induce DNA interstrand cross-linking in cell level, compound **17a** was selected as the representative compound for modified single cell gel electrophoresis assay (modified comet assay).[35,36] The DNA cross-linking agent melphalan was used as a positive control. The modified comet assay is based on the property of negatively charged DNA fragments migration when electric field is applied to the gel after cell lysis.[37] Human non-small lung carcinoma H1299 cells were treated with **17a** and melphalan at various concentrations breaks. The irradiated cells were then subjected to

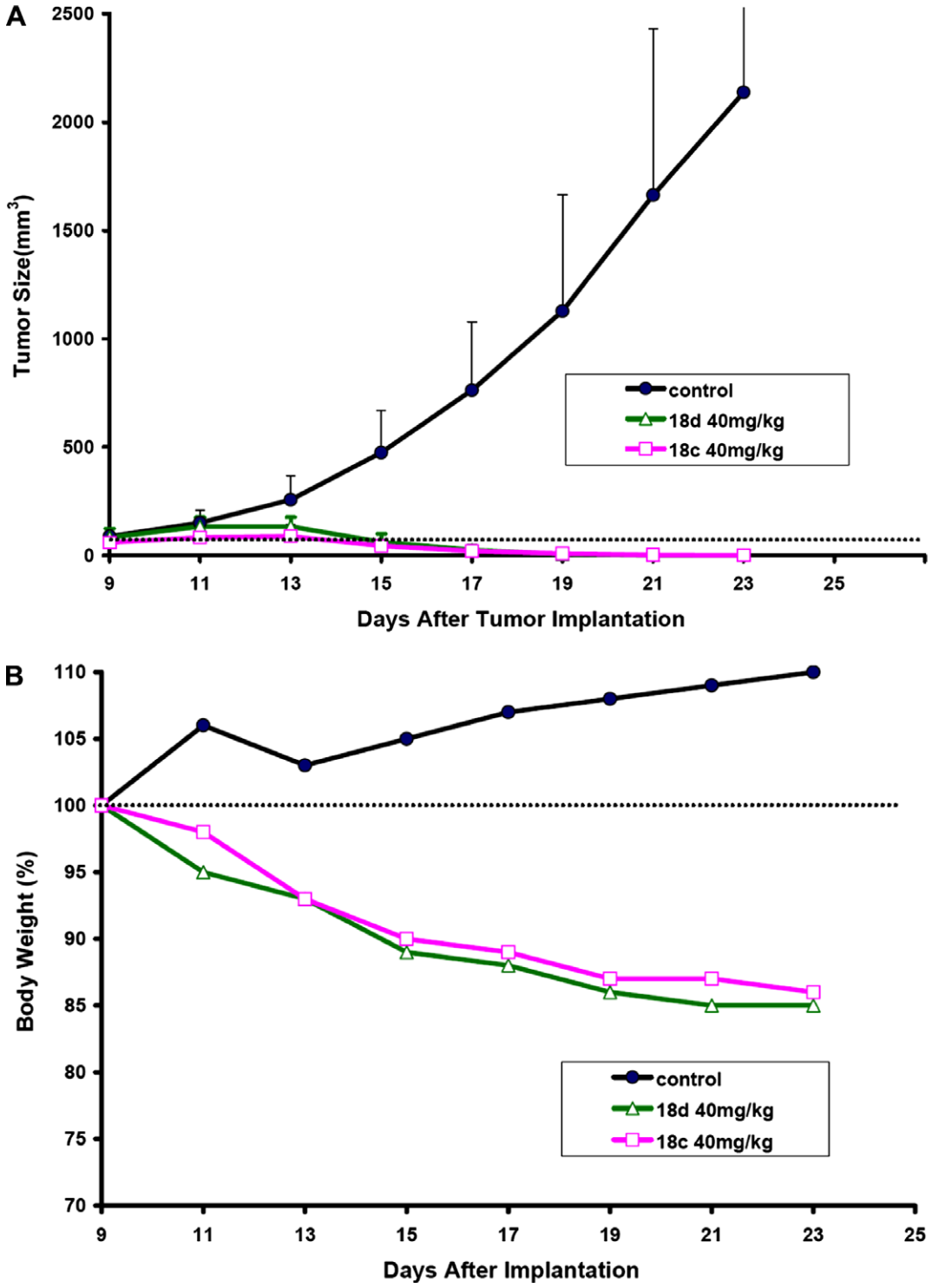


Figure 1. Therapeutic effect of *N*-mustard–quinoline conjugate having a hydrazinecarboxamide linker **18c**, 40 mg/kg, Q2D×6, iv injection, $n = 4$, $p < 0.0001$, –□–, CR on D19, D21, D21, and D23) and **18d** (40 mg/kg, Q2D×6, iv injection, $n = 4$, $p < 0.0001$, –Δ–, CR on D17, D19, D19, and D21) in nude mice bearing human mammary carcinoma MX-1 xenograft, control ($n = 4$, –●–); average tumor size changes (A) and average body weight changes (B).

modified comet assay. The results showed that **17a** was capable of inducing DNA interstrand cross-linking in a dose-dependent manner (Fig. 6). At the dose of 10 μM, this agent induced 31.3% DNA cross-linking, while melphalan induced 39.7% DNA cross-linking at the dose of 200 μM under the same experimental conditions. The results suggested that DNA interstrand cross-linking may be the main mechanism of action of **17a** and the related compounds.

3. Conclusion

Designing DNA-directed alkylating agents by linking the alkylating warhead to the DNA-affinic carrier is one of the promising strategies to overcome the drawbacks of alkylating agents. Recently, we have reported that *N*-mustard–9-anilinoacridine conjugates via a urea or carbamate linker exhibited potent cytotoxicity and therapeutic efficacy against various human tumor xenografts. Of these

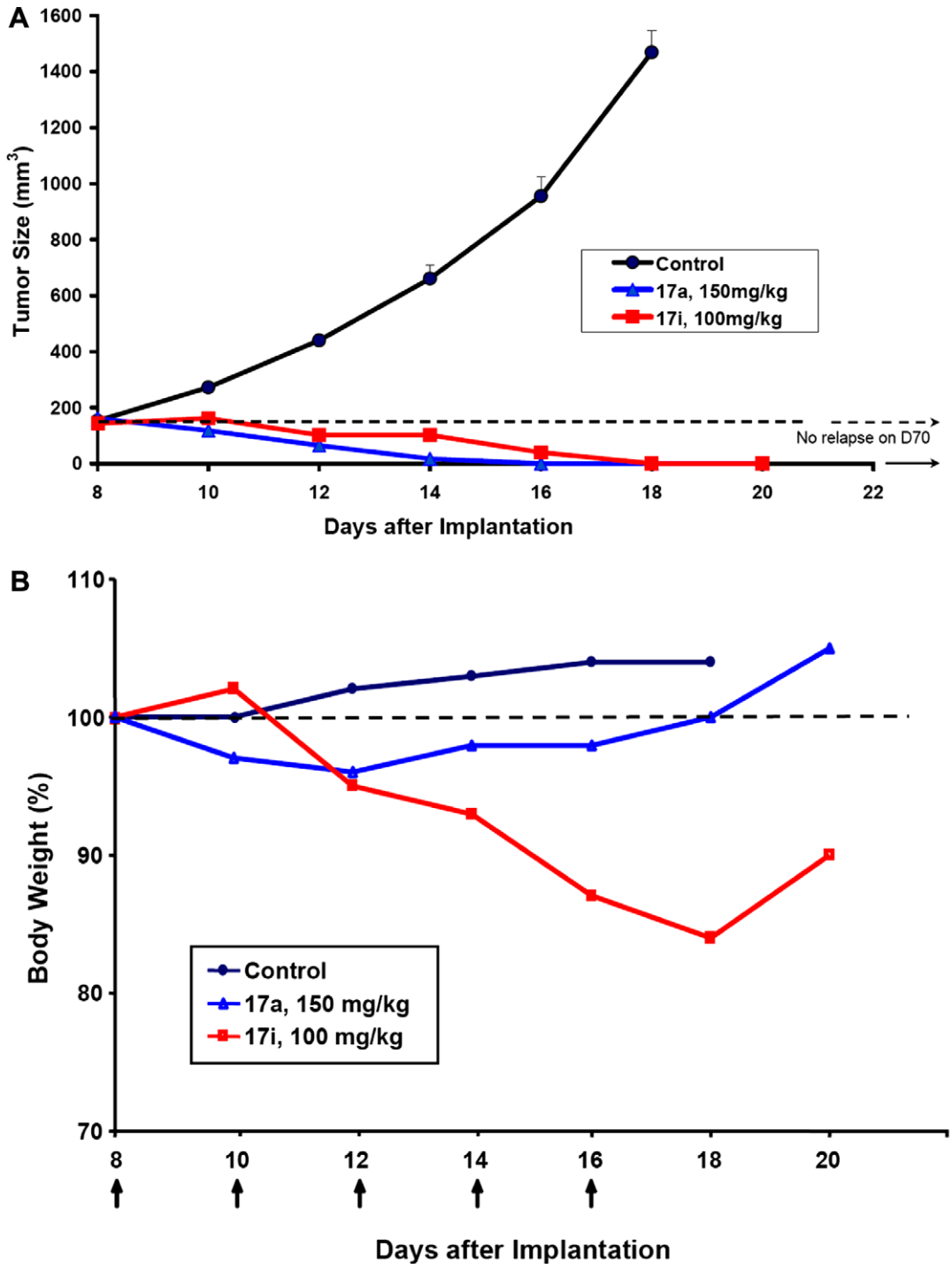


Figure 2. Therapeutic effect of *N*-mustard–quinoline conjugate having a urea linker **17a** (150 mg/kg, Q2D×5, iv injection, $n = 5$, $p < 0.0001$, 5/5 CR on D16, no relapse on D70, –▲–) and **17i** (100 mg/kg, Q2D×5, iv injection, $n = 5$, $p < 0.0001$, –■–, 5/5 CR on D18 and no relapse on D70) in nude mice bearing human mammary carcinoma MX-1 xenograft, control ($n = 5$, –●–); average tumor size changes (A) and average body weight changes (B).

conjugates, several derivatives are able to achieve complete tumor remission (CR) against breast carcinoma MX-1 xenograft in animal model with low toxicity. In the present study, we used quinoline chromophore as a carrier and synthesized a series of *N*-mustard–quinoline conjugates with a urea or hydrazinecarboxamide spacer. The newly synthesized conjugates also exhibited a broad spectrum of antitumor activity. Among human tumor xenografts tested, breast carcinoma MX-1 is the most sensitive to the test compounds. Complete tumor remission was achieved by **17a, i** and **18c, d**. The study of the comparable antitumor effects of *N*-mustard–quinoline conjugate (**17a**) and *N*-mustard–9-anilinoacrdine conjugate (BO-1037, **10**) against MX-1 xenograft in mice suggested that the former derivative is less potent than the later in terms of dose used, but **17a** is less toxic. Interestingly, the present investigation revealed that **17a** is as effective as gencitamine, but more potent than taxol and cyclophosphamide with less toxicity, in inhibiting human multidrug-resistant breast cancer MCF-7/Adr xenograft in mice. Furthermore, we have proved that DNA cross-linking probably is one of the major mechanisms of action of the newly synthesized conjugates by modified comet assay or by alkaline gel shift assay.

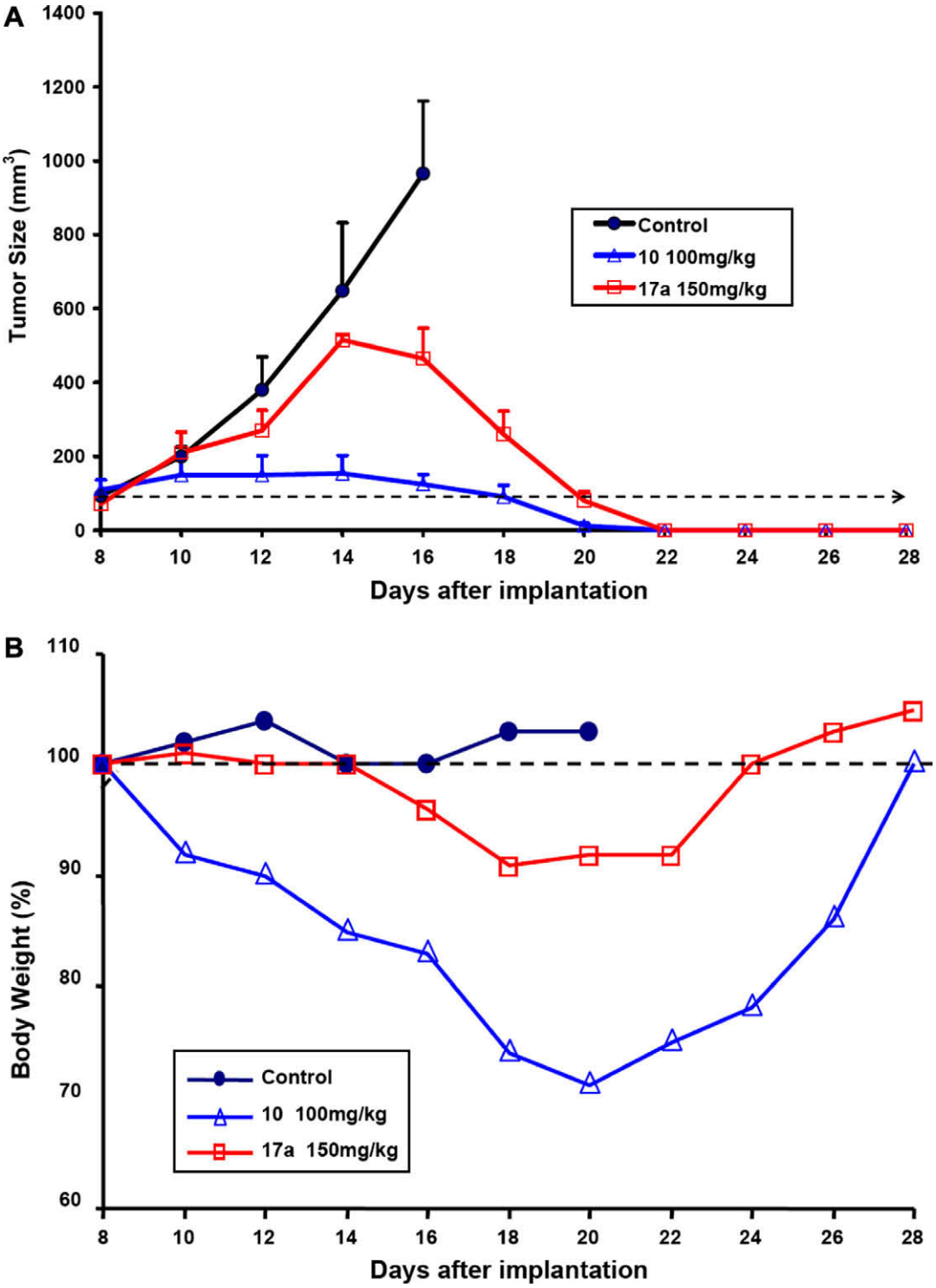


Figure 3. Therapeutic effect of *N*-mustard–quinoline conjugate having a urea linker **17a** (150 mg/kg, Q2D×5, 2 h iv infusion, $n = 4$, $p < 0.0001$, –□–) and *N*-mustard–AHMA conjugate having a urea linker **10** (100 mg/kg, Q2D×5, 2 h iv infusion, $n = 4$, $p < 0.0001$, –Δ–, CR on D22 and no relapse on D31) in nude mice bearing human mammary carcinoma MX-1 xenograft, control ($n = 4$, –●–); average tumor size changes (A) and average body weight changes (B).

In summary, the present study demonstrated that quinoline pharmacophore is also appropriate for using as a carrier in designing DNA-directed alkylating agents. In addition, we also found that conjugates using hydrazinecarboxamide as a linker result in increasing cytotoxic activity in comparison with the corresponding compounds bearing a urea spacer. This linker also can low the reactivity of the reactive *N*-mustard pharmacophore as we found that **17a** has a rather long half-life in rat plasma. Since the newly synthesized conjugates provide several compounds with potent in vivo antitumor activity, it will provide high possibility for us in finding a candidate for preclinical studies and eventually for clinical application.

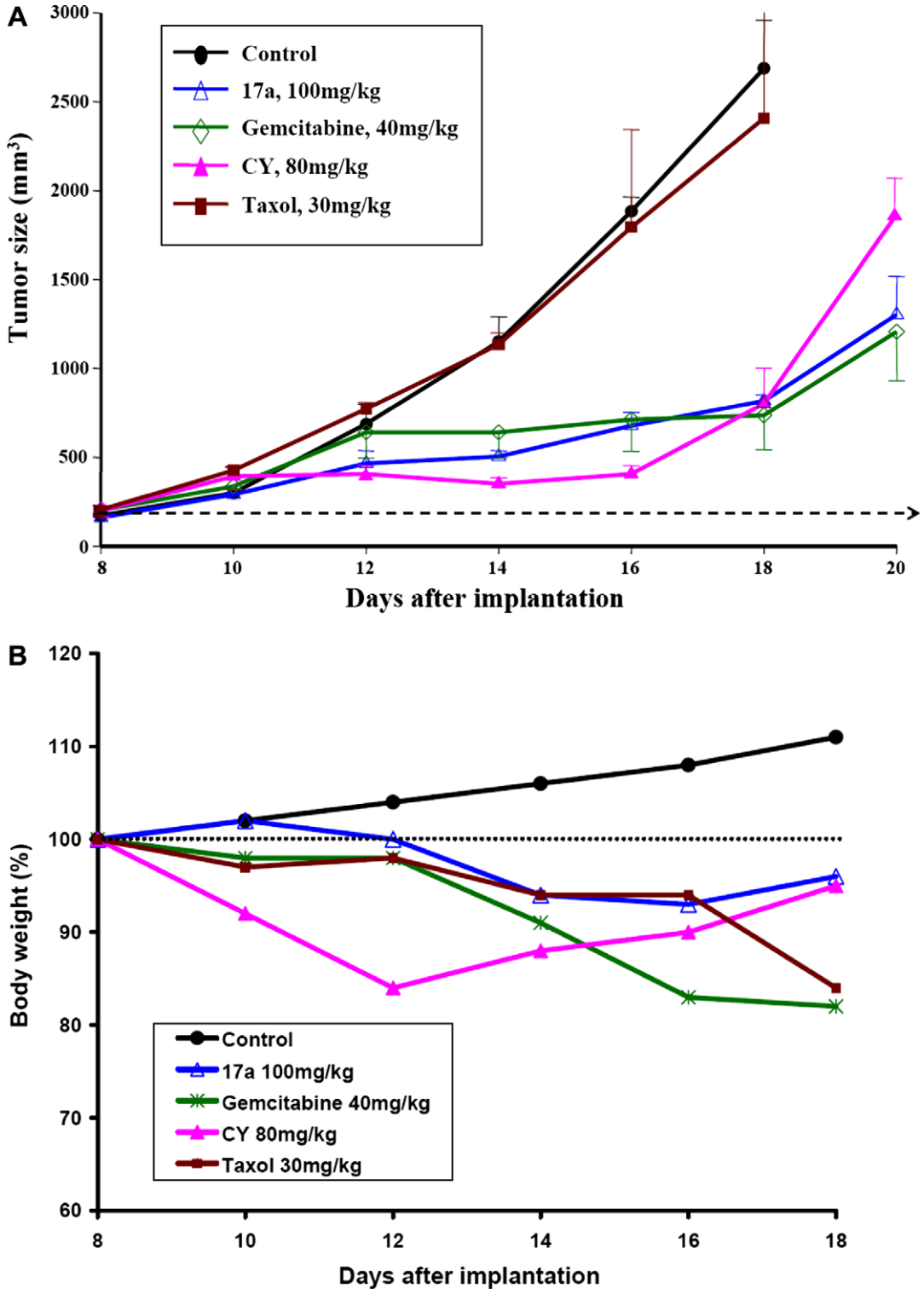


Figure 4. Therapeutic effect of *N*-mustard–quinoline conjugate having a urea linker **17a** (100 mg/kg, Q2D×6, iv injection, $n = 3$, $p < 0.0001$, –Δ–), Gemcitabine (40 mg/kg, Q2D×4, iv injection, $n = 3$, $p < 0.0001$, –◇–), Cyclophasmide (80 mg/kg, Q2D×3, iv injection, $n = 3$, $p < 0.0001$, –▲–), taxol (30 mg, Q2D×4, iv injection, $n = 3$, $p < 0.0001$, –■–) in nude mice bearing human breast MCF-7/Adr xenograft, control ($n = 3$, –●–); average tumor size changes (A) and average body weight changes (B).

4. Experimental section

4.1. 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,

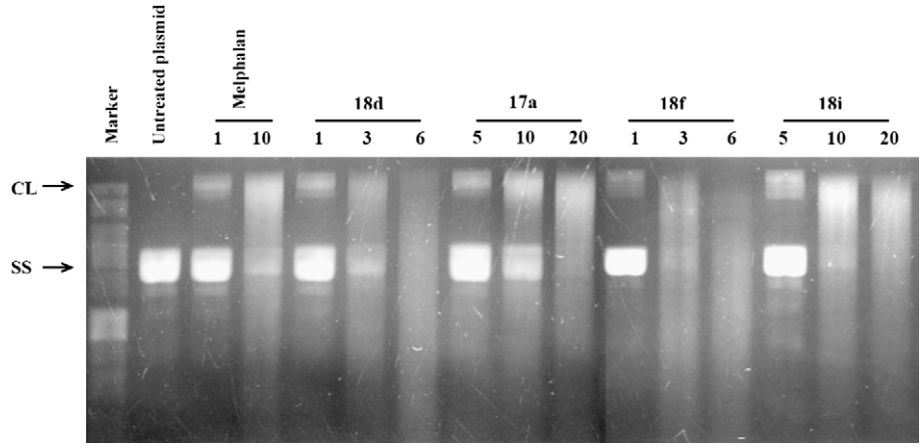


Figure 5. Representative DNA cross-linking gel shift assay for **17a**, **18d**, **18f**, and **18i** at various concentrations as indicated. Control lane shows single-stranded DNA (SL), while cross-linking (CL) shown in all tested lanes is DNA double-stranded cross-linking. melphalan (1 and 10 μM) was used as a positive control.

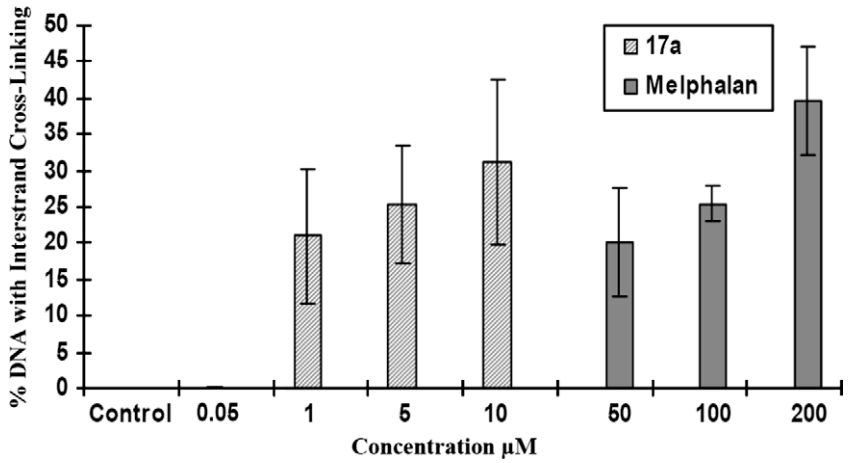


Figure 6. Induction of DNA interstrand cross-linking in H1299 cells by *N*-mustard–quinoline having a urea linker **17a** and melphalan measured by the modified comet assay. The experiments were performed in duplicate and repeated three times. Data points represent the mean ± SE of each test condition.

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 and 10% B in 25 min or 60% A and 40% B in 25 min (mobile phase A = acetonitrile, B = THF); flow rate, 1 mL/min; injected sample, 10 μL; column temp, 27 °C; wavelength, 254 nm. The purity of all compounds was $\geqslant$95% based on analytical HPLC.

4.2. Synthesis of 4-aminoquinolines (13b–m) and 4-hydrazinoquinoline (14a–d, f–m)

Detailed procedures for the synthesis of compounds **13b–m**, **14a–d**, **f–m** and intermediate **12b–m** along with their spectroscopic data are provided in the Supplementary data.

4.3. General procedure for the preparation of 17a–m

A solution of known 4-[*N*,*N*-bis(2-chloroethyl)amino]phenylisocyanate (**16**)[34] [freshly prepared from *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**15**,[15] 1.7 equiv) by treating with triphosgene (0.5 equiv) in the presence of TEA at −10 °C] in dry DMF was added dropwise to a solution of an appropriate 4-aminoquinoline (**13a–m**, 1.0 equiv) in dry DMF containing Et_3N (1.5–4 equiv) at room temperature. The reaction was monitored by thin-layer chromatography (SiO_2, solvent: $CHCl_3$/MeOH, about 10:1, v/v). After the completion of the reaction (3–5 h), the reaction mixture was evaporated to dryness in vacuo. The desired product was isolated and purified either by liquid column chromatography (SiO_2, solvent: $CHCl_3$) or by recrystallization.

4.3.1. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-(2-methyl-4-quinolinyl)urea (17a)

Compound **17a** was prepared from 4-aminoquinoline (**13a**, 0.47 g, 3 mmol) and isocyanate (**16**) [freshly prepared from *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**15**,[21] 1.68 g, 5.4 mmol)]. Yield, 1.06 g (85%); mp 174–175 °C; ^{1}H NMR ($CHCl_3$-d_6) δ 9.02 (br s, 1H, exchangeable, NH), 8.98 (br s, 1H, exchangeable, NH), 8.14 (s, 1H, ArH), 8.12 (s, 1H, ArH), 7.88 (d, J = 8.5 Hz, 1H, ArH), 7.77 (t, J = 4.5 Hz, 1H, ArH), 7.57 (t, 1H, ArH), 7.36 (d, J = 8.8 Hz, 2H, ArH), 6.56 (d, J = 7.9 Hz, 2H, ArH), 3.66 (t, J = 6.6 Hz, 4H, 2 × CH_2), 3.57 (t, J = 6.6 Hz, 4H, 2 × CH_2), 2.63 (s, 3H, Me). Anal. Calcd for ($C_{21}H_{22}Cl_2N_4O$): C, H, N.

4.3.2. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-(6-methoxy-2-methyl-4-quinolinyl) urea (17b)

Compound **17b** was prepared from 6-methoxy-2-methyl-4-quinolinamine (**13b**, 1.07 g, 5.31 mmol) and isocyanate **16**

(freshly prepared from **15**, 2.81 g, 9 mmol). Yield was 1.78 g (77%); mp 251–252 °C; ^{1}H NMR (DMSO-d_6) δ 9.31 (br s, 1H, NH, exchangeable), 9.21 (br s, 1H, NH, exchangeable), 8.17 (s, 1H, ArH), 7.82 (d, J = 9.1 Hz, 1H, ArH), 7.63 (s, 1H, ArH), 7.44 (dd, J = 2.3 and 9.1 Hz, 1H, ArH), 7.38 (d, J = 8.9 Hz, 2H, ArH), 6.76 (d, J = 8.9 Hz, 2H, ArH), 3.98 (s, 3H, OMe), 3.72–3.73 (m, 8H, 4 × CH_2), 2.58 (s, 3H, Me). Anal. Calcd for ($C_{22}H_{24}Cl_2N_4O_2$): C, H, N.

4.3.3. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-(6-methyl[1,3]-dioxolo[4,5-*g*] quinolin-8-yl)urea (17c)

Compound **17c** was prepared from 6-methyl[1,3]dioxolo-[4,5-*g*]quinolin-8-amine (**13c**, 1.01 g, 5 mmol) and isocyanate **16** (freshly prepared from **15**, 2.81 g, 9 mmol). Yield, 1.35 g (58%); mp 271–272 °C; ^{1}H NMR (DMSO-d_6) δ 2.54 (s, 3H, Me), 3.71 (m, 8H, 4 × CH_2), 6.23 (s, 2H, CH_2), 6.76 (d, J = 8.9 Hz, 2H, ArH), 7.25 (s, 1H, ArH), 7.35 (d, J = 8.9 Hz, 2H, ArH), 7.71 (s, 1H, ArH), 8.08 (s, 1H, ArH), 9.15. Anal. Calcd for ($C_{22}H_{22}Cl_2N_4O_3$): C, H, N.

4.3.4. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[6-(dimethyl-amino)-2-methyl-4-quinolinyl]urea (17d)

Compound **17d** was prepared from 4-amino-6-dimethylamino-2-methylquinoline (**13d**, 1.03 g, 5 mmol) and isocyanate **16** (freshly prepared from **15**, 2.89 g, 9 mmol). Yield, 1.53 g (65%); mp 267–268 °C; ^{1}H NMR (DMSO-d_6) δ 10.27 (1H, br s, NH, exchangeable), 9.98 (1H, br s, NH, exchangeable), 8.33 (s, 1H, ArH), 7.82 (d, J = 9.4 Hz, 1H, ArH), 7.55–7.58 (m, 1H, ArH), 7.44 (s, 1H, ArH), 7.40 (d, J = 8.9 Hz, 2H, ArH), 6.77 (d, J = 8.9 Hz, 2H, ArH), 3.72–3.73 (m, 8H, 4 × CH_2), 3.14 (s, 6H, 2 × Me), 2.66 (s, 3H, Me). Anal. Calcd for ($C_{23}H_{27}Cl_2N_5O$): C, H, N.

4.3.5. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[2-methyl-6-(1-pyrrolidinyl)-4-quinolinyl]urea (17e)

Compound **17e** was prepared from 2-methyl-6-(pyrrolidin-1-yl)-quinolin-4-amine (**13e**, 0.46 g, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.61 g (63%); mp 255–256 °C; ^{1}H NMR (DMSO-d_6) δ 11.83 (s, 1H, NH, exchangeable), 9.56 (s, 1H, NH, exchangeable), 7.70 (d, J = 8.8 Hz, 1H, ArH), 7.47 (d, J = 8.4 Hz, 2H, ArH), 7.16–7.05 (m, 1H, ArH), 7.05 (s, 1H, ArH), 6.76 (d, J = 8.8 Hz, 2H, ArH), 6.67 (s, 1H, ArH), 3.72 (m, 8H, 4 × CH_2), 3.34 (m, 4H, 2 × CH_2), 2.56 (s, 3H, Me), 2.00 (m, 4H, 2 × CH_2). Anal. Calcd for ($C_{25}H_{29}Cl_2N_5O$): C, H, N.

4.3.6. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-(2-phenyl-4-quinolinyl)urea (17f)

Compound **17f** was prepared from 2-phenylquinolin-4-amine (**13f**, 0.55 g, 2.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.40 g, 4.5 mmol). Yield, 0.87 g (73%); mp 194–195 °C; ^{1}H NMR (DMSO-d_6) δ 9.22 (s, 1H, NH, exchangeable), 9.07 (s, 1H, NH, exchangeable), 8.87 (s, 1H, ArH), 8.21 (d, J = 8.4 Hz, 1H, ArH), 8.15 (d, J = 7.6 Hz, 2H, ArH), 8.04 (d, J = 8.4 Hz, 1H, ArH), 7.79 (t, J = 7.6 Hz, 1H, ArH), 7.66 (t, J = 7.6 Hz, 1H, ArH), 7.58–7.55 (m, 2H, ArH), 7.52–7.50 (m, 1H, ArH), 7.29 (d, J = 8.8 Hz, 2H, ArH), 6.77 (d, J = 8.8 Hz, 2H, ArH), 3.72–3.69 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{26}H_{24}Cl_2N_4O$): C, H, N.

4.3.7. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-(6-phenyl[1,3]-dioxolo[4,5-*g*]quinolin-8-yl)urea (17g)

Compound **17g** was prepared from 6-phenyl-[1,3]dioxolo[4,5-*g*]quinolin-8-amine (**13g**, 0.66 g, 2.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.40 g, 4.5 mmol). Yield, 0.85 g (65%); mp 191–192 °C; ^{1}H NMR (DMSO-d_6) δ 8.92 (s, 1H, NH, exchangeable), 8.91 (s, 1H, NH, exchangeable), 8.71 (s, 1H, ArH), 8.08 (d, J = 7.2 Hz, 2H, ArH), 7.58 (s, 1H, ArH), 7.55–7.51 (m, 2H, ArH), 7.48–7.44 (m, 1H, ArH), 7.38–7.36 (m, 3H, ArH), 6.77 (d, J = 9.2 Hz, 2H, ArH), 6.25 (s, 2H, CH_2), 3.73–3.71 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{24}Cl_2N_4O_3$): C, H, N.

4.3.8. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[6-chloro-2-(3-methoxyphenyl)-4-quinolinyl] urea (17h)

Compound **17h** was prepared from 6-chloro-2-(3-methoxyphenyl)-4-quinolinamine (**13h**, 1.01 g, 3.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.71 g, 5.6 mmol). Yield, 1.50 g (78%); mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 9.21 (1H, s, NH, exchangeable), 8.98 (1H, s, NH, exchangeable), 8.88 (s, 1H, ArH), 8.30 (d, J = 1.8 Hz, 1H, ArH), 8.05 (d, J = 8.9 Hz, 1H, ArH), 7.78 (dd, J = 1.9 and 8.9 Hz, 1H, ArH), 7.71 (s, 1H, ArH), 7.67 (d, J = 7.8 Hz, 1H, ArH), 7.48 (t, J = 7.9 Hz, 1H, ArH), 7.38 (d, J = 8.9 Hz, 2H, ArH), 7.09 (dd, J = 2.3 and 8.2 Hz, 1H, ArH), 6.77 (d, J = 9.0 Hz, 2H, ArH), 3.87 (s, 3H, CH_3), 3.70–3.73 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{25}Cl_3N_4O_2$): C, H, N.

4.3.9. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[6-methoxy-2-(3-methoxy-phenyl)-4-quinolinyl]urea (17i)

Compound **17i** was prepared from 6-methoxy-2-(3-methoxyphenyl)-4-quinolinamine (**13i**, 0.56 mg, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.80 g (74%); mp 255–256 °C; ^{1}H NMR (DMSO-d_6) δ 9.01 (br s, 2H, exchangeable, 2 × NH), 8.78 (s, 1H, ArH), 7.99 (d, J = 10.3 Hz, 1H, ArH), 7.65 (m, 2H, ArH), 7.54 (m, 1H, ArH), 7.43 (m, 4H, ArH), 7.07 (dd, J = 2.2 and 8.1 Hz, 1H, ArH), 6.72 (d, J = 8.8 Hz, 2H, ArH), 4.01 (s, 3H, OMe), 3.87 (s, 3H, OMe), 3.72 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{28}H_{28}Cl_2N_4O_3$): C, H, N.

4.3.10. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[6-(3-methoxyphenyl) [1,3]dioxolo [4,5-*g*]quinolin-8-yl]urea (17j)

Compound **17j** was prepared from 6-(3-methoxy-phenyl)-[1,3]dioxolo[4,5-*g*]quinolin-8-amine (**13j**, 0.75 g, 2.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.40 g, 4.5 mmol). Yield, 0.95 g (69%); mp 202–203 °C; ^{1}H NMR (DMSO-d_6) δ 8.91 (s, 1H, NH, exchangeable), 8.89 (s, 1H, NH, exchangeable), 8.68 (s, 1H, ArH), 7.65–7.61 (m, 2H, ArH), 7.57 (s, 1H, ArH), 7.44 (t, J = 7.8 Hz, 1H, ArH), 7.38–7.36 (m, 3H, ArH), 7.04 (dd, J = 8 and 2.4 Hz, 1H, ArH), 6.76 (d, J = 8.8 Hz, 2H, ArH), 6.25 (s, 2H, CH_2), 3.86 (s, 3H, OMe), 3.73–3.72 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{28}H_{26}Cl_2N_4O_4$): C, H, N.

4.3.11. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[2-(3-methoxyphenyl)-6-(1-pyrrolidinyl)-4-quinolinyl]urea (17k)

Compound **17k** was prepared from 2-(3-methoxy-phenyl)-6-(pyrrolidin-1-yl)quinolin-4-amine (**13k**, 0.64 g, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.90 g (78%); mp 166–167 °C; ^{1}H NMR (DMSO-d_6) δ 9.17 (s, 1H, NH, exchangeable), 8.86 (s, 1H, NH, exchangeable), 8.70 (s, 1H, ArH), 7.88 (d, J = 9.2 Hz, 1H, ArH), 7.65–7.60 (m, 2H, ArH), 7.46–7.39 (m, 3H, ArH), 7.28 (d, J = 9.2 Hz, 1H, ArH), 7.02 (d, J = 8 Hz, 1H, ArH), 6.87 (s, 1H, ArH), 6.77 (d, J = 8.8 Hz, 2H, ArH), 3.86 (s, 3H, OMe), 3.73 (m, 8H, 4 × CH_2), 3.46 (m, 4H, 2 × CH_2), 2.07–2.05 (m, 4H, 2 × CH_2). Anal. Calcd for ($C_{31}H_{33}Cl_2N_5O_2$): C, H, N.

4.3.12. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*′-[6-(2-fluorophenyl)[1,3]dioxolo[4,5-*g*]quinolin-8-yl]urea (17l)

Compound **17l** was prepared from 6-(2-flourophenyl)-[1,3]dioxolo[4,5-*g*]quinolin-8-amine (**13l**, 0.71 g, 2.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.40 g, 4.5 mmol). Yield, 1.09 g (80%); mp 188–189 °C; ^{1}H NMR (DMSO-d_6) δ 8.92 (s, 1H, NH, exchangeable), 8.89 (s, 1H, NH, exchangeable), 8.59–8.58 (m, 1H, ArH), 7.99–7.94 (m, 1H, ArH), 7.59 (s, 1H, ArH), 7.53–7.48 (m, 1H, ArH), 7.38–7.32 (m, 5H, ArH), 6.76 (d, J = 9.2 Hz, 2H, ArH), 6.26 (s, 2H, CH_2), 3.72–3.71 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{23}Cl_2FN_4O_3$): C, H, N.

4.3.13. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-*N′*-[2-(2-fluorophenyl)-6-(1-pyrrolidinyl)-4-quinolinyl]urea (17m)

Compound **17m** was prepared from 2-(2-fluorophenyl)-6-(pyrrolidin-1-yl)quinolin-4-amine (**13m**, 0.85 g, 2.75 mmol) and isocyanate **16** (freshly prepared from **15**, 1.56 g, 5 mmol). Yield, 1.09 g (70%); mp 199–200 °C; ^{1}H NMR (DMSO-d_6) δ 9.03 (s, 1H, NH, exchangeable), 8.74 (s, 1H, NH, exchangeable), 8.58 (s, 1H, ArH), 7.99–7.96 (m, 1H, ArH), 7.87 (d, J = 9.6 Hz, 1H, ArH), 7.48–7.46 (m, 1H, ArH), 7.39–7.27 (m, 5H, ArH), 6.86 (s, 1H, ArH), 6.77 (d, J = 8.8 Hz, 2H, ArH), 3.72 (m, 8H, 4 × CH_2), 3.46 (m, 4H, 2 × CH_2), 2.08–2.06 (m, 4H, 2 × CH_2). Anal. Calcd for ($C_{30}H_{30}Cl_2FN_5O$): C, H, N.

4.4. General procedure for the preparation of 18a–d, f–m

A solution of known 4-[*N*,*N*-bis(2-chloroethyl)amino]phenylisocyanate (**16**)[34] [freshly prepared from *N*,*N*-bis(2-chloroethyl)benzene-1,4-diamine hydrochloride (**15**,[15] 1.5 equiv) by treating with triphosgene (0.5 equiv) in the presence of TEA at −10 °C] in dry DMF was added dropwise to a solution of appropriate 4-hydrazinoquinolines (**14a–m**, 1.0 equiv) in dry DMF containing Et_3N (1.5–4 equiv) at room temperature. The reaction was monitored by thin-layer chromatography (SiO_2, solvent: $CHCl_3$/MeOH, about 10:1, v/v). After the completion of the reaction (1.5–4 h), the reaction mixture was evaporated to dryness in vacuo. The desired products **18a–d**, **f–m** were purified by triturating with ethyl acetate and chloroform, respectively.

4.4.1. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-(2-methyl-4-quinolinyl)-hydrazinecarboxamide (18a)

Compound **18a** was prepared from 4-hydrazino-2-methylquinoline (**14a**, 1.05 g, 5 mmol) and isocyanate **16** (freshly prepared from **15**, 2.60 g, 8 mmol). Yield, 2.05 g (94%); mp 263–264 °C; ^{1}H NMR (DMSO-d_6) δ 10.84 (br s, 1H, NH, exchangeable), 9.12 (1H, s, NH, exchangeable), 9.04 (1H, s, NH, exchangeable), 8.49 (d, J = 8.5 Hz, 1H, ArH), 8.03 (d, J = 8.5 Hz, 1H, ArH), 7.97 (t, J = 7.1 Hz, 1H, ArH), 7.72 (t, J = 7.7 Hz, 1H, ArH), 7.31 (d, J = 9.0 Hz, 2H, ArH), 6.89 (s, 1H, ArH), 6.70 (d, J = 9.0 Hz, 2H, ArH), 3.68–3.71 (m, 8H, 4 × CH_2), 2.71 (s, 3H, CH_3). Anal. Calcd for ($C_{21}H_{23}Cl_2N_4O$): C, H, N.

4.4.2. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-(6-methoxy-2-methyl-4-quinolinyl)-hydrazinecarboxamide (18b)

Compound **18b** was prepared from 4-hydrazino-6-methoxy-2-methylquinoline (**14b**, 0.36 g, 1.5 mmol) and isocyanate **16** (freshly prepared from **15**, 0.84 g, 2.7 mmol). Yield, 0.413 g (60%); mp 255–256 °C; ^{1}H NMR (DMSO-d_6) δ 10.65 (br s, 1H, NH, exchangeable), 9.11 (1H, s, NH, exchangeable), 9.01 (1H, s, NH, exchangeable), 7.96 (d, J = 9.2 Hz, 1H, ArH), 7.91 (d, J = 2.2 Hz, 1H, ArH), 7.61 (dd, J = 2.2 and 9.2 Hz, 1H, ArH), 7.31 (d, J = 9.0 Hz, 2H, ArH), 6.84 (s, 1H, ArH), 6.70 (d, J = 9.0 Hz, 2H, ArH), 3.94 (s, 3H, OMe), 3.68–3.70 (m, 8H, 4 × CH_2), 2.68 (s, 3H, Me). Anal. Calcd for ($C_{22}H_{25}Cl_2N_5O_2$): C, H, N.

4.4.3. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-(6-methyl[1,3]-dioxolo[4,5-*g*]quinolin-8-yl)-hydrazinecarboxamide (18c)

Compound **18d** was prepared from 8-hydrazino-6-methyl-[1,3]dioxolo[4,5-*g*]quinoline (**14c**, 0.51 g, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.76 g (80%); mp 253–254 °C; ^{1}H NMR (DMSO-d_6) δ 10.42 (br s, 1H, NH, exchangeable), 9.06 (1H, s, NH, exchangeable), 9.06 (1H, s, NH, exchangeable), 7.44 (s, 1H, ArH), 7.86 (s, 1H, ArH), 6.78 (s, 1H, ArH), 7.29 (d, J = 8.1 Hz, 2H, ArH), 6.69 (d, J = 8.1 Hz, 2H, ArH), 6.32 (s, 2H, CH_2), 3.68–3.70 (m, 8H, 4 × CH_2), 2.63 (s, 3H, Me). Anal. Calcd for ($C_{22}H_{23}Cl_2N_5O_3$): C, H, N.

4.4.4. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[6-(dimethylamino)-2-methyl-4-quinolinyl]-hydrazinecarboxamide (18d)

Compound **18d** was prepared from 4-hydrazino-*N*,*N*,2-trimethyl-6-quinolinamine (**14d**, 1.26 g, 5 mmol) and isocyanate **16** (freshly prepared from **15**, 2.60 g, 8 mmol). Yield, 1.61 g (68%); mp 260–261 °C; ^{1}H NMR (DMSO-d_6) δ 10.42 (br s, 1H, NH, exchangeable), 9.10 (1H, s, NH, exchangeable), 8.94 (1H, s, NH, exchangeable), 7.85 (d, J = 9.3 Hz, 1H, ArH), 7.57 (dd, J = 9.4 and 2.1 Hz, 1H, ArH), 7.30–7.32 (m, 3H, ArH), 6.69–6.74 (m, 3H, ArH), 3.68–3.70 (m, 8H, 4 × CH_2), 3.07 (s, 6H, 2 × CH_3), 2.64 (s, 3H, CH_3). Anal. Calcd for ($C_{23}H_{28}Cl_2N_6O$): C, H, N.

4.4.5. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-(2-phenyl-4-quinolinyl)-hydrazinecarboxamide (18f)

Compound **18f** was prepared from 4-hydrazinyl-2-phenylquinoline (**14f**, 1.4 g, 5 mmol) and isocyanate **16** (freshly prepared from **15**, 2.60 g, 8 mmol). Yield, 1.48 g (60%); mp 235–236 °C; ^{1}H NMR (DMSO-d_6) δ 11.13 (s, 1H, NH, exchangeable), 9.31 (s, 1H, NH, exchangeable), 9.28 (s, 1H, NH, exchangeable), 8.59 (d, J = 8.4 Hz, 1H, ArH), 8.34 (d, J = 8.4 Hz, 1H, ArH), 8.06–8.02 (m, 3H, ArH), 7.77 (t, J = 7.6 Hz, 1H, ArH), 7.72–7.66 (m, 3H, ArH), 7.31–7.29 (d, J = 8.8 Hz, 2H, ArH), 7.19 (s, 1H, ArH), 6.70 (d, J = 8.8 Hz, 2H, ArH), 3.71–3.67 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{26}H_{25}Cl_2N_5O$): C, H, N.

4.4.6. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-(6-phenyl[1,3]-dioxolo[4,5-*g*]quinolin-8-yl)-hydrazinecarboxamide (18g)

Compound **18g** was prepared from 8-hydrazinyl-6-phenyl-[1,3]dioxolo[4,5-*g*]quinoline (**14g**, 0.77 g, 2.75 mmol) and isocyanate **16** (freshly prepared from **15**, 1.56 g, 5 mmol). Yield, 0.95 g (64%); mp 289–291 °C; ^{1}H NMR (DMSO-d_6) δ 10.59 (s, 1H, NH, exchangeable), 9.19 (s, 1H, NH, exchangeable), 9.15 (s, 1H, NH, exchangeable), 7.97–7.95 (m, 3H, ArH), 7.77 (s, 1H, ArH), 7.66–7.65 (m, 3H, ArH), 7.29 (d, J = 8.8 Hz, 2H, ArH), 7.10 (s, 1H, ArH), 6.69 (d, J = 8.8 Hz, 2H, ArH), 6.37 (s, 2H, CH_2), 3.70–3.68 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{25}Cl_2N_5O_3$): C, H, N.

4.4.7. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[6-chloro-2-(3-methoxyphenyl)-4-quinolinyl]-hydrazinecarboxamide (18h)

Compound **18h** was prepared from 6-chloro-4-hydrazinyl-2-(3-methoxyphenyl)quinoline (**14h**, 0.75 g, 4 mmol) and isocyanate **16** (freshly prepared from **15**, 2.24 g, 7.2 mmol). Yield, 0.86 g (61%); mp 237–238 °C; ^{1}H NMR (DMSO-d_6) δ 11.22 (s, 1H, NH, exchangeable), 9.36 (s, 1H, NH, exchangeable), 9.25 (s, 1H, NH, exchangeable), 8.77 (d, J = 2 Hz, 1H, ArH), 8.47 (d, J = 9.2 Hz, 1H, ArH), 8.12–8.08 (dd, J = 2.2 and 9.0 Hz, 1H, ArH), 7.63–7.54 (m, 3H, ArH), 7.31–7.23 (m, 4H, ArH), 6.70 (d, J = Hz, 2H, ArH), 3.90 (s, 3H, OMe), 3.69–3.68 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{26}Cl_3N_5O_2$): C, H, N.

4.4.8. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[6-methoxy-2-(3-methoxyphenyl)-4-quinolinyl]-hydrazinecarboxamide (18i)

Compound **18i** was prepared from 4-hydrazinyl-6-methoxy-2-(3-methoxyphenyl)quinoline (**14i**, 0.59 g, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 2.7 mmol). Yield, 0.79 g (71%); mp 215–216 °C; ^{1}H NMR (DMSO-d_6) δ 10.89 (s, 1H, NH, exchangeable), 9.23 (s, 2H, 2 × NH, exchangeable), 8.29 (d, J = 9.2 Hz, 1H, ArH), 7.99 (s, 1H, ArH), 7.67 (d, J = 8.8 Hz, 1H, ArH), 7.57–7.50 (m, 3H, ArH), 7.31–7.23 (m, 3H, ArH), 7.16 (s, 1H, ArH), 6.69 (d, J = 8.4 Hz, 2H, ArH), 3.97 (s, 3H, OMe), 3.89 (s, 3H, OMe), 3.68 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{28}H_{29}Cl_2N_5O_3$): C, H, N.

4.4.9. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[6-(3-methoxyphenyl)-[1,3]dioxolo [4,5-*g*]quinolin-8-yl]-hydrazinecarboxamide (18j)

Compound **18j** was prepared from 8-hydrazinyl-6-(3-methoxyphenyl)-[1,3]dioxolo[4,5-*g*]quinoline (**14j**, 0.62 g, 2 mmol) and iso-

cyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.83 g (73%); mp 279–280 °C; 10.60 (s, 1H, NH, exchangeable), 9.26 (s, 1H, NH, exchangeable), 9.22 (s, 1H, NH, exchangeable), 7.99 (s, 1H, ArH), 7.89 (s, 1H, ArH), 7.57–7.49 (m, 3H, ArH), 7.29 (d, J = 8.4 Hz, 2H, ArH), 7.21 (d, J = 7.6 Hz, 1H, ArH), 7.10 (s, 1H, ArH), 6.69 (d, J = 8.4 Hz, 2H, ArH), 6.35 (s, 2H, CH_2), 3.89 (s, 3H, OMe), 3.69–3.67 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{28}H_{27}Cl_2N_5O_4$): C, H, N.

4.4.10. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[2-(3-methoxyphenyl)-6-(1-pyrrolidinyl)-4-quinolinyl]-hydrazinecarboxamide (18k)

Compound **18k** was prepared from 4-hydrazinyl-2-(3-methoxyphenyl)-6-(pyrrolidin-1-yl)quinoline (**14k**, 1.17 g, 3.5 mmol) and isocyanate **16** (freshly prepared from **15**, 1.6 g, 5.4 mmol). Yield, 1.41 g (68%); mp 190–191 °C; 10.60 (s, 1H, NH, exchangeable), 9.26 (s, 1H, NH, exchangeable), 9.20 (s, 1H, NH, exchangeable), 8.19 (d, J = 8.8 Hz, 1H, ArH), 7.55–7.53 (m, 2H, ArH), 7.49–7.43 (m, 2H, ArH), 7.30 (d, J = 8 Hz, 2H, ArH), 7.23–7.21 (m, 2H, ArH), 7.06 (s, 1H, ArH), 6.69 (d, J = 8.4 Hz, 2H, ArH), 3.89 (s, 3H, OMe), 3.69–3.68 (m, 8H, 4 × CH_2), 3.40 (m, 4H, 2 × CH_2), 2.04 (m, 4H, 2 × CH_2). Anal. Calcd for ($C_{31}H_{34}Cl_2N_6O_2$): C, H, N.

4.4.11. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[6-(2-fluorophenyl)[1,3]dioxolo[4,5-*g*]quinolin-8-yl]-hydrazinecarboxamide (18l)

Compound **18l** was prepared from 6-(2-fluorophenyl)-8-hydrazinyl-[1,3]dioxolo[4,5-g]quinoline (**14l**, 0.56 g, 2 mmol) and isocyanate **16** (freshly prepared from **15**, 1.21 g, 3.6 mmol). Yield, 0.87 g (78%); mp 211–212 °C; 10.70 (s, 1H, NH, exchangeable), 9.19 (s, 1H, NH, exchangeable), 9.16 (s, 1H, NH, exchangeable), 7.95 (s, 1H, ArH), 7.84 (t, J = 6.8 Hz, 1H, ArH), 7.72–7.68 (m, 1H, ArH), 7.65 (s, 1H, ArH), 7.53–7.45 (m, 2H, ArH), 7.28 (d, J = 8.8 Hz, 1H, ArH), 7.03 (s, 1H, ArH), 6.69 (d, J = 8.8 Hz, 2H, ArH), 6.37 (s, 1H, ArH), 6.37 (s, 2H, CH_2), 3.69–3.67 (m, 8H, 4 × CH_2). Anal. Calcd for ($C_{27}H_{24}Cl_2FN_5O_3$): C, H, N.

4.4.12. *N*-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[2-(2-fluorophenyl)-6-(1-pyrrolidinyl)-4-quinolinyl]-hydrazinecarboxamide (18m)

Compound **18m** was prepared from 2-(2-fluorophenyl)-4-hydrazinyl-6-(pyrrolidin-1-yl)quinoline (**14m**, 1.29 g, 4 mmol) and isocyanate **16** (freshly prepared from **15**, 2.24 g, 7.2 mmol). Yield, 1.44 g (62%); mp 270–271 °C; ^{1}H NMR (DMSO-d_6) δ 10.67 (s, 1H, NH, exchangeable), 9.26 (s, 1H, NH, exchangeable), 9.20 (s, 1H, NH, exchangeable), 8.06 (d, J = 9.2 Hz, 1H, ArH), 7.83 (t, J = 7.6 Hz, 1H, ArH), 7.72–7.67 (m, 1H, ArH), 7.52–7.45 (m, 3H, ArH), 7.30–7.28 (m, 3H, ArH), 6.96 (s, 1H, ArH), 6.69 (d, J = 8.8 Hz, 2H, ArH), 3.69–3.68 (m, 8H, 4 × CH_2), 3.42 (m, 4H, 2 × CH_2), 2.05 (m, 4H, 2 × CH_2). Anal. Calcd for ($C_{30}H_{31}Cl_2FN_6O$): C, H, N.

4.5. Biological experiments

4.5.1. Cytotoxicity assays

The cytotoxic effects of the newly synthesized compounds were determined in T-cell acute lymphocytic leukemia (CCRF-CEM) and their resistant subcell lines (CCRF-CEM/Taxol and CCRF-CEM/VBL) by the XTT assay[38] and human solid tumor cells (i.e., breast carcinoma MX-1 and colon carcinoma HCT-116) by the SRB assay[39] in a 72 h incubation using a microplate spectrophotometer as described previously.[40] After the addition of phenazine methosulfate–XTT solution, incubated at 37 °C for 6 h and absorbance at 450 and 630 nm was detected on a microplate reader (EL 340). The cytotoxicity of the newly synthesized compounds against non-small cell lung cancer H1299, human prostate cancer PC-3, human lung adenocarcinoma CL 1-0 and CL 1-5 and human breast adenocarcinoma MCF-7 were determined by the Alamar blue assay[41] in a 72 h incubation using a microplate spectrophotometer as described previously. 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[42] based on the median-effect principle and plot.[43,44] Ranges given for taxol, vinblastine and cisplatin were mean ± SE (n = 4).

4.5.2. 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. Compounds were administered via the tail vein for iv injection as described previously.[40] 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 refers to total weight minus the weight of the tumor. 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.

4.5.3. 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 **17a**, **18d** and **18f**, and **18i** 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 reaction, the plasmid DNA was linearized by digestion with *Bam*HI 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(1000 ng) 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.

4.5.4. Determination of DNA interstrand cross-linking by modified single cell gel electrophoresis (comet) assay

The level of DNA interstrand cross-linking was determined using a modified comet assay.[35,36] 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 (**17a** or melphalan). After being treated with 1 h, the cells were exposed to 20 Gy X-ray to induce DNA strand breaks. An aliquot of 5×10^5 cells was suspended in 50 μL of phosphate-buffered saline, mixed with 250 μL of 1.2% low melting point agarose, and subjected to comet assay. The tail moments of 100 cells were analyzed for each treatment using the COMET assay software. The degree of DNA interstrand cross-linking presented in a drug-treated sample was determined by comparing the tail moment of the irradiated control, which was calculated by the following formula;

% of DNA with interstrand cross-linking

$$\left\{1 - \left(\frac{\mathrm{TMdi} - \mathrm{TMcu}}{\mathrm{TMci} - \mathrm{TMcu}}\right)\right\} \times 100$$

where TMdi = mean tail moment of drug treated, irradiated sample, TMcu = mean tail moment of unirradiated control sample, TMci = mean tail moment of irradiated control sample.

4.5.5. Determination of half-life of 17a in rat plasma

The chromatographic system consisted of a photodiode-array system, chromatographic pump, an auto-sampler equipped with a 40 μL sample loop. Compound **17a** was separated from rat plasma using a revise phase column (30 × 150 mm, particle size 5 μm) maintained at an ambient temperature (25±1 °C) to perform the ideal chromatographic system. The detector wavelength was set at 266 nm. The mobile phase was comprised of 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 0.5 mL/min and the samples were quantified using peak area. An aliquot of plasma sample (40 μL, with **17a** 5 μg/mL) was vortex-mixed with acetonitrile (1:2, v/v) for protein precipitation and centrifuged at 10,000*g* for 10 min. The supernatant was passed through a 0.2 μm filter for injection into the HPLC.

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Supplementary data

Supplementary data (detailed experimental procedures for the synthesis of intermediate compounds **12b–m**, **13b–m**, **14a–d**, **f–m** along with their spectroscopic data, in vivo figures 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.2010.01.061.

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