European Journal of Medicinal Chemistry 76 (2014) 155–169

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design and synthesis of potent antitumor water-soluble phenyl N-mustard-benzenealkylamide conjugates via a bioisostere approach

Satishkumar D. Tala [a], Tai-Hsin Ou [a,b], Yi-Wen Lin [a], Kiranben S. Tala [a], Shu-Hsin Chao [a], Ming-Hsi Wu [a], Tung-Hu Tsai [d], Rajesh Kakadiya [a], Sharda Suman [a], Ching-Huang Chen [a], Te-Chang Lee [a,b,*], Tsann-Long Su [a,c,*]

[a] Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan
[b] Institute of Pharmacology, National Yang-Ming University, Taipei 11221, Taiwan
[c] Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 40402, Taiwan
[d] Institute of Traditional Medicine, National Yang-Ming University, Taipei 11221, Taiwan

ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form
31 December 2013
Accepted 8 February 2014
Available online 11 February 2014

Keywords:
Water-soluble antitumor agents
Cytotoxicity
Cell cycle
Apoptosis
Combination therapy
DNA cross-linking

ABSTRACT

A series of new, water-soluble phenyl N-mustard-benzenealkylamide conjugates containing hydrophilic ω-dialkylaminoalkylamide or ω-cyclic aminoalkylamide moieties were synthesized via a bioisostere approach. These compounds have a broad spectrum of antitumor activity against a panel of human tumor cell lines. Of these derivatives, compound **18b** effectively suppressed the growth of colon cancer (HCT-116), prostate cancer (PC3), and lung cancer (H460) xenografts. The growth of HCT-116 xenografts was almost completely suppressed when co-treated with compound **18b** and 5-fluorouracil. Furthermore, compound **18b** can induce DNA cross-linking and cell-cycle arrest at the G2/M phase. Early preclinical studies, including pharmacokinetics in rats, inhibition of the hERG, and 14 days of acute intravenous injection toxicity, suggest that compound **18b** is a promising candidate for further preclinical studies.

1. Introduction

We have previously designed and synthesized a variety of DNA-directed alkylating agents by linking an alkyl or phenyl N-mustard pharmacophore to DNA-binding molecules (such as 9-anilinoacridines, acridines, and quinolines) via a urea, carboxamide, or hydrazinecarboxamide linker [1–3]. These linkers are able to lower the chemical reactivity of the N-mustard pharmacophore; as a result, these agents are chemically stable with good pharmacokinetic (PK) profiles in rat plasma. However, the main challenge of these conjugates is their poor water solubility. The water solubility of a drug candidate is a critical factor in the drug development process because it can influence the evaluation of a drug's antitumor efficacy, PK profile, and toxicity/safety in animal models. Therefore, water solubility is a decisive factor in the successful development of a drug candidate for clinical application.

One strategy for improving the water solubility of a drug candidate while preserving its biological potency is the introduction of a hydrophilic side chain. For example, topotecan (**1**, Fig. 1) [4] and irinotecan (**2**) [5], which are derived from camptothecin, bear a tertiary amino functional group that significantly improves their water solubility by forming acid salts. The acid salt of bendamustine (**3**) is also water soluble and is currently used for the treatment of patients with chronic lymphocytic leukemia (Hodgkin's disease and non-Hodgkin's lymphoma) [6]. Another strategy is to prepare a

Abbreviations: HCT-116, human colon cancer; PC3, human prostate cancer; H460, human lung cancer; (5-FU), 5-fluorouracil; MX-1, human breast cancer; EA, ethyl acetate; TEA, triethylamine; CCRF-CEM, human lymphoblastic leukemia; CCRF-CEM/VBL, human lymphoblastic leukemia resistant to vinblastine; H1299, human lung cancer; H3347, human colon cancer; DLD-1, human colon cancer; HT-29, human colon cancer; QD ×6, once per day for 6 days; Q7D ×2, every 7 days for 2 doses; Q2D ×5, every 2 days for 5 doses; Q4D ×2, every 4 days for 2 doses; CI, combination index; Q3D ×3, every 3 days for 3 doses; PARP, poly ADP-ribose polymerase; c-caspase-3, cleaved caspase-3; c-PARP, cleaved poly ADP-ribose polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; FP, fluorescence polarization; D5W, 5% dextrose isotonic solution; PI, propidium iodide; NBF, neutral buffered formalin.

* Corresponding authors. Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan.
E-mail addresses: bmtcl@ibms.sinica.edu.tw (T.-C. Lee), tlsu@ibms.sinica.edu.tw (T.-L. Su).

http://dx.doi.org/10.1016/j.ejmech.2014.02.018
0223-5234/

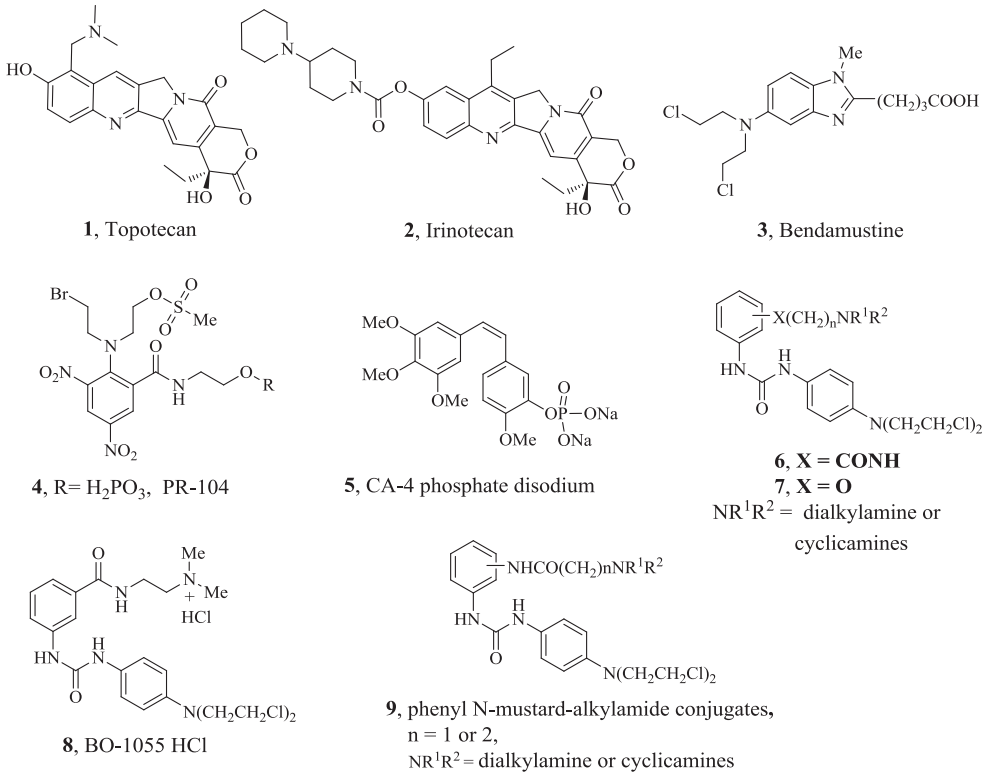


Fig. 1. Chemical structures of some DNA alkylating agents.

sodium phosphate salt. For example, PR-104 (**4**) is a novel prodrug of dinitrobenzamide mustard [7], which is currently in Phase II clinical trials for the treatment of various cancers. Similarly, combretastatin A-4 phosphate disodium salt (CA-4-P, **5**) was synthesized to form a water-soluble prodrug of the anti-microtubule agent combretastatin A-4 [8].

To overcome the poor water solubility of N-mustard derivatives, we have recently synthesized a series of phenyl N-mustard-benzamide conjugates containing an ureido linker. A variety of hydrophilic side chains (such as ω-*N*,*N*-dialkylaminoalkyl or ω-cyclicaminoalkyl side chains) were linked to the benzene ring via a carboxamide or ether linkage at the *meta*- or *para*-position of the ureido linker (**6** and **7**, respectively, Fig. 1). The tertiary amino functionality on the side chain can be converted into a variety of water-soluble salts with inorganic or organic acids [9]. Although compounds bearing a hydrophilic carboxamide side chain (**6**) can form water-soluble hydrochloride salts, compounds bearing a hydrophilic alkoxy ether side chain (**7**) do not have improved water solubility, even as the HCl salt. Of these derivatives, the water-soluble compound BO-1055 hydrochloride (**8**) exhibited significant cytotoxicity against a variety of human tumor cell lines and possessed potent therapeutic efficacy against a variety of human solid tumor cells [such as human breast cancer (MX-1), colon cancer (HCT-116), and prostate cancer (PC3)] in xenograft models. Our unpublished results indicate that the therapeutic window of compound **8** is rather narrow; the maximum tolerated dose (MTD) is approximately 30–40 mg/kg, while the lethal dose (LD_{50}) of 14-day intravenous injection (iv inj) acute toxicity in mice is approximately 76 mg/kg. Accordingly, there is an urgent need for lead optimization to enhance the antitumor activity and minimize the toxicity of compound **8** via structure modification.

The concept of bioisosterism is often applied to lead optimization in new drug research and development. For example, the inversion of ester or carboxamide functionalities is one strategy for the design of new drugs using a bioisostere approach [10–12]. Applying this approach to the design of new water-soluble N-mustard derivatives with improved antitumor activity, we inverted the carboxamide functionality of the ω-*N*,*N*-dialkylaminoalkyl- or ω-cyclic aminoalkyl-benzamide moieties (Fig. 2a) to *N*-phenyl ω-dialkylaminolalkylamide or ω-cyclic aminoalkylamide (Fig. 2b). The hydrophilic side chains on the benzene ring remain at the *meta*- or *para*-position of the ureido linker through an alkylamide linkage. The newly synthesized conjugates (**9**, Fig. 1) were water-soluble and were subjected to antitumor studies. The results of the present study demonstrate that the structure–activity relationship (SAR) of the newly synthesized phenyl N-mustard-benzenealkylamide conjugates is quite different from that of the phenyl N-mustard-benzamide compounds. Based on the in vitro cytotoxicity and the antitumor efficacy in human tumor xenograft models of the newly synthesized compounds, compound **18b** (phenyl N-mustard-benzenealkylamide derivative) is superior to compound **8** (phenyl N-mustard-benzamide derivative) against the colon cancer cell line HCT-116 and the lung cancer cell line H460, while displaying reduced toxicity. Compound **18b** was selected for early preclinical studies. Here, we report the chemical synthesis,

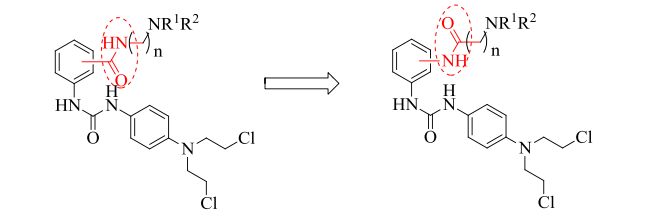


Fig. 2. Drug design of new derivatives using a bioisostere approach.

antitumor evaluation, and mechanism of action of these newly synthesized water-soluble phenyl N-mustard-benzenealkylamide derivatives and early preclinical studies of this candidate compound.

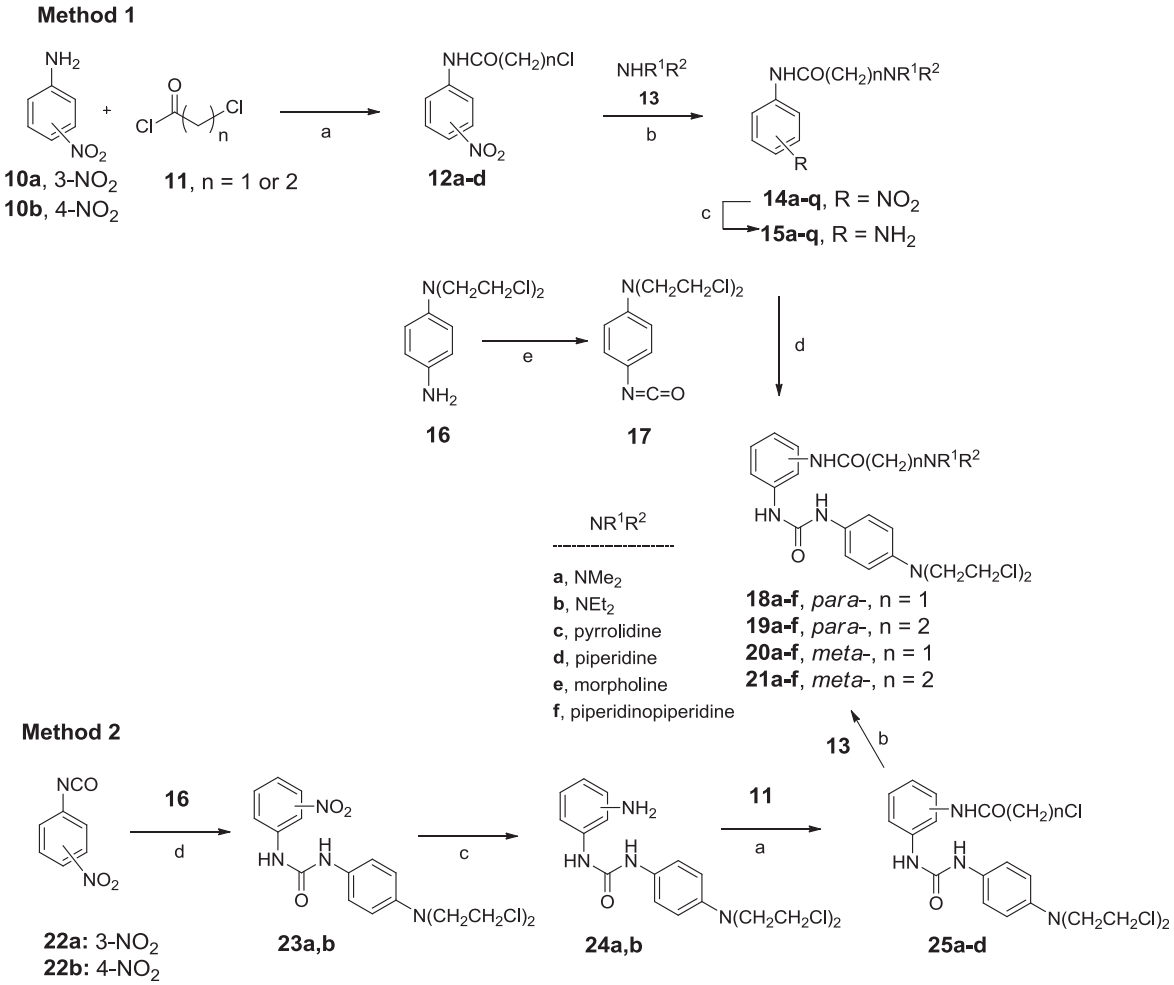
2. Results and discussion

2.1. Chemistry

The new phenyl N-mustard-benzenealkylamide conjugates (Fig. 2b) were synthesized by two synthetic routes (Methods 1 and 2), as depicted in Scheme 1. **Method 1:** The commercially available 3- or 4-nitroaniline (**10**) was used as the starting material. Reaction of **10** with chloroacetyl chloride or 3-chloropropionyl chloride (**11**, $n = 1$ or 2, respectively) in tetrahydrofuran (THF) at room temperature (rt) afforded compounds **12** according to the procedure described in the literature [13]. Reaction of **12** with various *N*,*N*-dialkylamines or cyclic amines (**13**) yielded compounds **14** (NR^1R^2 = NMe_2, NEt_2, pyrrolidine, piperidine, morpholine, or piperidinopiperidine). The nitro compounds **14** were then reduced to the corresponding amine derivatives **15** by catalytic hydrogenation (10% Pd/C, H_2) in ethyl acetate (EA). Condensation of **15** with the known 4-[*N*,*N*-bis(2-chloroethyl) amino]phenylisocyanate (**17**) [9] [freshly prepared by reacting **16** with triphosgene in anhydrous dimethylformamide (DMF) in the presence of triethylamine (TEA)] afforded the desired compounds **18, 19,** and **21** in fair yields (approximately 40%) after purification by column chromatography. To improve the yields of the final products, an efficient synthetic route with higher yield (Method 2) was developed.

Method 2: Reacting the commercially available 3- or 4-nitrophenyl isocyanate (**22**) with phenyl N-mustard amine **16** in the presence of TEA yielded **23** (Scheme 1). The nitro derivatives **23** were then reduced to the corresponding amine derivatives **24** by catalytic hydrogenation (10% Pd/C, H_2) in EA. Compounds **24** were reacted with ω-haloalkyl carboxylic acid chlorides (**11**) in THF at rt to afford compounds **25** ($n = 1$ or 2), which were then reacted with various *N*,*N*-dialkylamines or cyclic amines (**13**) to give the desired target compounds **20** and **21f**. The yields and the physical properties of all synthesized compounds (Table S1) are provided as Supplementary material.

Method 2 facilitates the preparation and purification of the final products with higher yields compared to Method 1. Because compounds **18a** and **18b** exhibited potent antitumor activity, we synthesized these two compounds by both Methods. Consistently, both derivatives were more efficiently prepared from compound **25** with higher yield (76 and 84%, respectively) by Method 2 than from compounds **15f** and **15g** by Method 1 (46 and 48%, respectively), suggesting that Method 2 can be applied as an optimized process for large-scale preparation of the desired compounds.



Scheme 1. Synthesis of water-soluble phenyl *N*-mustard-benzenealkylamide conjugates. Reagents and conditions: (a) THF, rt; (b) Different secondary amine, THF, 50 °C; (c) 10% Pd/C, H_2, 35-38 psi, EA; (d) TEA, rt; (e) triphosgene, TEA, DMF, or $CHCl_3$.

2.2. Biological activity

2.2.1. In vitro cytotoxicity

The antiproliferative activities of the newly synthesized conjugates were evaluated against human lymphoblastic leukemia (CCRF-CEM and its vinblastine-resistant sub-cell line CCRF-CEM/VBL), human prostate cancer (PC3), human lung cancer (H460 and H1299), and human colon cancer (HCT-116, H3347, DLD-1 and HT-29) cell lines in vitro. The SARs of these derivatives were assessed based on their cytotoxicities against CCRF-CEM and CCRF-CEM/VBL cells in culture (Table 1).

For the series of compounds possessing a hydrophilic side chain at the *para*-position of the ureido linker, there was no apparent difference in potency against CCRF-CEM cell growth among compounds bearing an acetamide (**18a**–**f**, $n = 1$) or propanamide (**19a**–**f**, $n = 2$) side chain. However, the cytotoxicity of these compounds decreased when the size of the tertiary amine moiety increased. Intriguingly, the acetamide derivatives **18a**–**f** were more active than propanamide conjugates **19a**–**f** against CCRF-CEM/VBL cells, a multidrug-resistant (MDR) cell line. As for the compounds bearing a hydrophilic side chain at the *meta*-position of the ureido linker, compounds with a propanamide side chain (**21a**–**f**) were generally more cytotoxic than the corresponding compounds bearing an acetamide side chain (**20a**–**f**). However, the size of the tertiary amine moiety had no apparent effect on their activities. Similarly, compounds **20a**–**f** were more active than compounds **21a**–**f** against MDR cells. Comparing the cytotoxicities of the *para*- and *meta*-substituted conjugates, the *para*-substituted derivatives (**18a**–**f** and **19a**–**f**) were more potent than the corresponding *meta*-substituted derivatives (**20a**–**f** and **21a**–**f**) at inhibiting CCRF-CEM cell growth in vitro. However, the *meta*-substituted derivatives (**20a**–**f** and **21a**–**f**) were generally more active than the corresponding *para*-substituted derivatives (**18a**–**f** and **19a**–**f**) against MDR cells.

As for the antiproliferative effects of the newly synthesized conjugates against a variety of human solid tumor cell lines [e.g., prostate cancer (PC3), lung cancer (H460 and H1299), and colon cancer (HCT-116)], the *para*-substituted derivatives (**18a**–**f** and **19a**–**f**) were generally more active than the corresponding *meta*-substituted derivatives (**20a**–**f** and **21a**–**f**) (Table 1). For both *para*- and *meta*-substituted compounds, the length of the side-chain ($n = 1$ or 2) did not influence their cytotoxicities. However, bulky tertiary amine groups, such as morpholine or 4-piperidinopiperidine, greatly decreased their potency against cancer cells. As shown in Table 1, the *para*-substituted derivatives (**18a**–**f** and **19a**–**f**) were most cytotoxic to HCT-116 cancer cells, followed by H460, PC3, and H1299 cells. Notably, most of the *para*-substituted derivatives were more cytotoxic than irinotecan in inhibiting HCT-116 cell growth in vitro. Therefore, we selected compounds **18a**–**d** and **19a**–**d** and studied their cytotoxicities against three additional human colon cell lines (e.g., H3347, DLD-1, and HT-29 cells). Among the colon tumor cell lines tested, HCT-116 and H3347 cells were most sensitive to the tested compounds (Table 2).

To compare the antitumor efficacies of the phenyl N-mustard-benzamides and phenyl N-mustard-benzenealkylamides, we selected six compounds from the phenyl N-mustard-benzamide series and assessed their cytotoxicity using the same bioassay (Alamar Blue assay) [14]. The IC_{50} values of these compounds are summarized in Table 3. Based on their IC_{50} values (Table 1 and 3), the tested compounds from the phenyl N-mustard-benzamide series are apparently less potent than the corresponding phenyl N-

Table 1
The cytotoxicities of the new water-soluble phenyl N-mustard-benzenealkylamide conjugates against the growth of a variety of human tumor cell lines in vitro.

Compd	IC_{50} (μM)[a]					
	CCRF-CEM	CCRF-CEM/VBL[b]	PC3	H460	H1299	HCT-116
18a	0.29 ± 0.04	1.34 ± 0.40 [4.62×][c]	7.33 ± 0.28	1.21 ± 0.17	5.36 ± 0.53	0.85 ± 0.41
18b	0.32 ± 0.05	2.16 ± 1.51 [6.75×]	3.57 ± 0.46	0.93 ± 0.17	3.43 ± 0.33	0.82 ± 0.33
18c	0.26 ± 0.05	2.00 ± 0.96 [7.69×]	3.52 ± 0.23	0.95 ± 0.14	3.41 ± 0.28	1.33 ± 0.25
18d	0.31 ± 0.02	1.45 ± 0.40 [4.68×]	2.24 ± 0.19	1.05 ± 0.10	2.52 ± 0.12	0.99 ± 0.08
18e	1.13 ± 0.20	18.38 ± 2.75 [16.27×]	16.0 ± 0.60	4.74 ± 0.46	18.63 ± 1.263	6.67 ± 1.87
18f	1.14 ± 0.17	52.21 ± 12.31 [45.8×]	7.24 ± 1.13	14.93 ± 1.31	14.36 ± 1.33	4.24 ± 1.01
19a	0.25 ± 0.06	8.03 ± 0.05 [32.12×]	3.08 ± 0.42	1.41 ± 0.18	7.62 ± 1.07	1.77 ± 0.24
19b	0.30 ± 0.08	8.27 ± 0.13 [27.57×]	2.47 ± 0.39	1.83 ± 0.19	10.30 ± 0.35	3.08 ± 1.70
19c	0.28 ± 0.05	17.12 ± 0.38 [61.14×]	3.48 ± 0.14	1.03 ± 0.23	6.43 ± 0.65	2.87 ± 1.84
19d	0.24 ± 0.03	6.20 ± 1.46 [25.83×]	2.28 ± 0.19	1.58 ± 0.37	3.03 ± 0.05	2.41 ± 0.72
19e	1.06 ± 0.05	23.42 ± 3.52 [22.09×]	9.74 ± 0.14	3.95 ± 1.63	15.19 ± 0.64	7.02 ± 0.42
19f	0.72 ± 0.12	119.64 ± 3.96 [166.17×]	29.96 ± 8.63	14.46 ± 0.84	36.01 ± 3.17	11.07 ± 0.96
20a	0.78 ± 0.19	2.70 ± 0.12 [3.46×]	19.01 ± 2.27	8.74 ± 0.72	10.64 ± 0.47	9.56 ± 0.70
20b	2.01 ± 0.92	1.38 ± 0.45 [0.69×]	12.99 ± 0.73	5.67 ± 0.35	11.69 ± 0.38	4.62 ± 1.09
20c	3.41 ± 1.13	0.97 ± 0.09 [0.28×]	12.85 ± 0.54	6.55 ± 0.87	9.36 ± 0.53	5.41 ± 1.12
20d	1.35 ± 0.63	1.33 ± 0.34 [0.99×]	10.08 ± 0.57	5.62 ± 1.16	8.55 ± 0.26	7.50 ± 0.21
20e	3.87 ± 0.70	1.32 ± 0.73 [0.34×]	35.78 ± 6.38	19.50 ± 2.94	26.42 ± 1.72	12.96 ± 2.38
20f	30.43 ± 1.44	1.81 ± 0.12 [0.06×]	25.91 ± 0.73	12.28 ± 0.71	12.80 ± 0.77	6.15 ± 1.16
21a	0.52 ± 0.05	14.39 ± 0.52 [27.67×]	13.49 ± 1.75	16.79 ± 1.95	8.99 ± 0.80	3.28 ± 0.12
21b	0.86 ± 0.15	13.04 ± 3.45 [15.62×]	12.97 ± 1.65	6.79 ± 0.45	5.77 ± 0.83	3.21 ± 0.54
21c	0.67 ± 0.05	27.08 ± 4.68 [40.42×]	10.34 ± 2.21	9.84 ± 0.30	4.33 ± 0.46	5.11 ± 0.07
21d	0.39 ± 0.20	6.01 ± 1.53 [15.41×]	5.73 ± 0.23	7.88 ± 0.15	4.63 ± 0.48	3.51 ± 0.13
21e	3.10 ± 0.14	4.84 ± 1.16 [1.56×]	35.45 ± 1.00	38.47 ± 0.75	15.94 ± 0.27	16.75 ± 0.21
21f	1.63 ± 0.35	1.53 ± 0.43 [0.94×]	22.09 ± 2.49	14.22 ± 0.74	11.04 ± 0.45	15.66 ± 1.34
Vinblastine[d]	1.30 ± 0.18	670 ± 40 [511×]	ND[e]	ND	ND	ND
vincristine[d]	2.32 ± 0.29	2128.0 ± 248.86 [917×]	ND	ND	ND	ND
Cisplatin	3.54 ± 0.66	1.64 ± 0.16 [0.46×]	9.96 ± 0.85	18.41 ± 3.02	13.573 ± 1.183	12.69 ± 1.83
Irinotecan	ND	ND	ND	ND	ND	4.05 ± 0.72

[a] Data represent the mean ± STDEV of three to six independent experiments for each compound.
[b] CCRF-CEM/VBL is a sub-cell line of CCRF-CEM that is 511-fold more resistant to vinblastine compared to the IC_{50} of the parent cell line.
[c] Numbers in brackets are resistance factors determined by comparison with the corresponding IC50 of the parent cell line.
[d] IC_{50} values in nM.
[e] Not determined.

Table 2
The cytotoxicities of the selected phenyl N-mustard-benzenealkylamide conjugates against various human colon (H3347, DLD-1, and HT-29) cell lines in culture.

Compd	IC_{50} (μM)[a]		
	H3347	DLD-1	HT-29
18a	1.53 ± 0.39	5.76 ± 2.09	20.05 ± 0.90
18b	0.87 ± 0.09	3.85 ± 1.17	19.05 ± 1.94
18c	1.53 ± 1.13	4.37 ± 0.30	12.26 ± 0.39
18d	1.16 ± 0.20	4.53 ± 1.59	11.84 ± 0.69
19a	2.86 ± 0.70	9.84 ± 2.72	23.63 ± 0.09
19b	1.03 ± 0.41	7.82 ± 1.12	19.34 ± 6.42
19c	3.68 ± 0.70	13.9 ± 0.67	14.14 ± 5.55
19d	2.41 ± 0.92	6.50 ± 0.31	12.27 ± 8.56
Oxaliplatin	4.69 ± 0.74	58.05 ± 11.96	21.29 ± 1.34
5-FU	11.64 ± 1.77	29.14 ± 1.32	24.87 ± 2.19
Irinotecan	7.53 ± 1.13	25.10 ± 3.16	82.75 ± 0.95

[a] Data represent the mean ± STDEV of three to six independent experiments for each compound.

mustard-benzenealkylamide series compounds (e.g., **19a** > **26**; **19c** > **27**; **19d** > **28**; **21a** > **8**; **21c** > **29**; and **21d** > **30**). These results indicate that the newly synthesized phenyl N-mustard-benzenealkylamide derivatives are more cytotoxic and have greater efficacy against MDR cells than the corresponding conjugates in the phenyl N-mustard-benzamide series.

2.2.2. *In vivo antitumor activity*

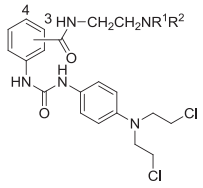
We selected compounds **18a** and **18b** for studying their therapeutic efficacy against various human solid tumors in xenograft models based on the in vitro cytotoxicities of the newly synthesized derivatives. Compound **8**, which was previously selected as a lead compound for early preclinical antitumor studies, was included for comparison. Nude mice bearing various tumors (subcutaneously implanted) were treated with the tested compounds via iv injection. The results are summarized in Table 4. Treatment of human colon cancer HCT-116 xenograft-bearing nude mice with compounds **18a**, **18b**, **8**, and irinotecan at 30 mg/kg once per day for 6 consecutive days (QD ×6) resulted in approximately 51, 84, 79, and 85% tumor suppression, respectively. These results indicate that compound **18b** is as effective as irinotecan but more potent than compounds **18a** and **8**. The average body weight of all the treated mice recovered after cessation of treatment (Table 4), suggesting that the toxicity of the tested compounds to the hosts is acceptable based on the average body weight loss.

We also evaluated the therapeutic effects of compound **18b** against a human colon cancer H3347 xenograft in animal models and compared that to oxaliplatin and irinotecan. As shown in Table 4, compound **18b** suppresses tumor growth as potently as irinotecan (>90% tumor suppression on D31) at the same treatment regime (30 mg/kg, QD ×6) but is more potent than oxaliplatin, which induced approximately 69% tumor suppression at a dose of 15 mg/kg every 7 days for two doses (Q7D ×2). Moreover, based on the average body weight loss, none of the drugs examined displayed significant toxicity to the host at the treated dosages.

The therapeutic efficacy of compound **18b** was further investigated in mice bearing PC3 tumor xenografts. Compound **8** and carboplatin were used for comparison. As shown in Table 4, the PC3 xenograft-bearing mice were treated with compound **18b** with various doses and schedules, such as 30 mg/kg, QD ×6, and 40 or 50 mg/kg, every 2 days for 5 doses (Q2D ×5). The therapeutic efficacy of compound **18b** apparently increased in a dose-dependent manner. At a dose of 50 mg/kg (Q2D ×5), compound **18b** is as potent as carboplatin (100 mg/kg, Q7D ×2) in suppressing the growth of PC3 cells. Nevertheless, compound **8** (40 mg/kg, Q2D ×5), which caused approximately 89% tumor suppression on D38, was the most potent agent against PC3 cells.

For the treatment of lung cancer H460 xenograft-bearing nude mice, compound **18b** (30 mg/kg, QD ×6) induced more than 53% tumor suppression on the day of sacrifice, which is slightly more potent than cisplatin [6 mg/kg, every 4 days for 2 doses (Q4D ×2)], which induced 48% tumor suppression (Table 4). However, compound **8** had very little effect on the inhibition of H460 in xenograft models, indicating that the therapeutic efficacy of compound **18b** is superior to that of compound **8**. Based on the average body weight loss, compound **18b** is less toxic than other investigated compounds. The graphical presentation of the therapeutic efficacy of compounds **18a** and **18b** in nude mice bearing human colon cancer (HCT-116 and H3347), prostate cancer (PC3), and lung cancer (H460) xenografts is provided in Fig. S1–S4.

Table 3
The cytotoxicity of water-soluble phenyl N-mustard-benzamide conjugates against various human tumor cell lines in vitro.



Compd	Position	NR^1R^2	IC_{50} (μM)[a]					
			CCRF-CEM	CCRF-CEM/VBL	PC3	HCT-116	H460	H1299
26	4	NMe_2	0.57 ± 0.03	106.23 ± 4.00 [186.47×]	6.25 ± 0.25	5.64 ± 0.70	31.64 ± 1.96	31.98 ± 0.95
27	4		0.56 ± 0.01	132.83 ± 1.59 [237.20×]	4.53 ± 0.22	6.48 ± 0.08	17.82 ± 1.87	18.54 ± 1.18
28	4		0.43 ± 0.04	48.83 ± 1.25 [113.58×]	4.25 ± 0.09	5.25 ± 0.52	9.44 ± 0.35	7.58 ± 0.24
8	3	NMe_2	1.23 ± 0.26	1.05 ± 0.12 [0.85×]	17.64 ± 2.88	4.85 ± 0.71	38.27 ± 1.53	14.79 ± 1.39
29	3		0.53 ± 0.02	126.36 ± 4.58 [238.42×]	6.54 ± 0.28	4.90 ± 0.66	34.15 ± 1.62	18.66 ± 0.15
30	3		0.57 ± 0.01	28.45 ± 3.59 [49.91×]	4.54 ± 0.12	5.47 ± 0.31	12.82 ± 0.20	10.28 ± 0.27

[a] Data represent the mean ± STDEV of three to six independent experiments for each compound.

Table 4
The therapeutic efficacy of compounds **18a** and **18b** in nude mice bearing human colon cancer (HCT-116 and H3347), prostate cancer (PC3), and lung cancer (H460) xenografts.

Compd	Tumor xenograft	Dosage (mg/kg)	Schedule (iv injection)	Tumor suppression[a]		Average body weight change		Animal survival (n/n)
				Maximum (%)	On day sacrificed (%)	BWLmax[b] (%)[b]	On day sacrificed (%)	
18b	HCT-116	30	QD ×6	91% (Day 15)	84% (Day 31)	−3 (Day 13)	+9 (Day 31)	4/4
18a	HCT-116	30	QD ×6	72% (Day 13)	51% (Day 30)	−5 (Day 13)	+3 (Day 30)	4/4
8	HCT-116	30	QD ×6	81% (Day 15)	79% (Day 31)	−7 (Day 13)	0 (Day 31)	4/4
Irinotecan	HCT-116	30	QD ×6	92% (Day 15)	85% (Day 31)	−3 (Day 13)	+8 (Day 31)	4/4
18b	H3347	30	QD ×6	94% (Day 15)	90% (Day 31)	−2 (Day 10)	+10 (Day 31)	5/5
Irinotecan	H3347	30	QD ×6	90% (Day 16)	90% (Day 31)	0[c]	+8 (Day 31)	5/5
Oxaliplatin	H3347	15	Q7D ×2	58% (Day 13)	69% (Day 31)	−2 (Day 13)	+11 (Day 31)	5/5
18b	PC3	30	QD ×6	56% (Day 21)	16% (Day 38)	−15 (Day 13)	−28[d] (Day 31)	5/5
18b	PC3	40	Q2D ×5	68% (Day 21)	3% (Day 38)	−15 (Day 16)	−12[d] (Day 38)	5/5
18b	PC3	50	Q2D ×5	88% (Day 24–28)	62% (Day 38)	−23 (Day 16)	−8 (Day 38)	4/4
8	PC3	40	Q2D ×5	94% (Day 28–31)	89% (Day 38)	−16 (Day 13)	−2 (Day 38)	4/4
Carboplatin	PC3	100	Q7D ×2	88% (Day 24)	64% (Day 38)	−29 (Day 16)	−7 (Day 38)	4/4
18b	H460	30	QD ×6	61% (Day 19–26)	53% (Day 37)	−18 (Day 15)	−5 (Day 37)	5/5
8	H460	30	QD ×6	17% (Day 15)	8% (Day 37)	−16 (Day 15)	−6 (Day 37)	5/5
Cisplatin	H460	6	Q4D ×2	71% (Day 15)	47% (Day 37)	−14 (Day 15)	−5 (Day 37)	4/4

[a] Antitumor effect [(1−T/C) × 100].
[b] BWLmax = Maximum average body weight loss on day indicated.
[c] No body weight loss.
[d] Tumor growth led to body weight decreases in the control and drug-treated groups.

2.2.3. Combination therapy of compound 18b+5-FU against human colon cancer HCT-116

Combination therapy has become a standard regimen for the treatment of patients with colon cancer. FOLFOX, a combination of folinic acid, 5-fluorouracil (5-FU), and oxaliplatin, is the most often used combination therapy in patients with advanced colon cancer [15]. The studies described above indicate that compound **18b** is more effective than compound **8** against colon cancer HCT-116 xenografts in animal models. Consequently, we investigated the therapeutic effects of the combination of compound **18b** and 5-FU (**18b**+5-FU) against HCT-116 cells. After treatment of HCT-116 cells with various concentrations of compound **18b**, 5-FU, and **18b**+5-FU in cell culture, the combination index (CI) value of each drug was determined using CompuSyn software (version 1.0.1; CompuSyn, Inc., Paramus, NJ) [16]. A drug combination is considered to be synergistic if its CI value is significantly below 1 [17]. As shown in Fig. 3, the combination of compound **18b** and 5-FU at ratios of 1:3 and 1:6 induced synergistic cytotoxicity to HCT-116 cells.

To investigate the antitumor efficacy of a combination regime of **18b**+5-FU, HCT-116 xenograft-bearing mice were treated with **18b**, 5-FU, oxaliplatin, **18b**+5-FU, and oxaliplatin+5-FU. As shown in Fig. 4, co-treatment of HCT-116 xenograft-bearing mice with compound **18b** (20 or 30 mg/kg, QD ×6) and 5-FU [75 mg/kg, Q7D ×2, via intraperitoneal injection (ip inj)] achieved 92% and 95% tumor reduction on D35, respectively. However, approximately 83, 74, and 54% tumor suppression was observed when the tumor-bearing mice were treated with oxaliplatin [15 mg/kg every 3 days for 3 doses (Q3D ×3)]+5-FU (75 mg/kg, Q7D ×2, ip), oxaliplatin (15 mg/kg Q3D ×3), or 5-FU (75 mg/kg, Q7D ×2, ip), respectively. These results demonstrate that the therapeutic effect of **18b**+5-FU is superior to that of oxaliplatin+5-FU, oxaliplatin alone, or 5-FU alone. It should be noted that 2 of 5 mice died on day 28 when mice were co-treated with oxaliplatin+5-FU, indicating that the combination of compound **18b**+5-FU is less toxic to the host and may have great potential benefit for the treatment of advanced colon cancer.

2.2.4. DNA cross-linking study

We used an alkaline agarose gel shift assay to study whether the newly synthesized compounds can cross-link with DNA. The linearized pEGFP-N1 DNA was treated with compounds **18a** and **18b** at various concentrations (1, 2, and 4 μM). Melphalan (1 and 10 μM) was used as a positive control. As shown in Fig. 5A, compounds **18a** and **18b** efficiently formed DNA interstrand cross-links in a dose-dependent manner. These results suggest that the DNA cross-linking capabilities of the tested compounds correlate with their in vitro cytotoxicity and are the main mechanism by which they inhibit cancer cell growth. We further performed a DNA modified comet assay [18,19] to investigate whether the newly synthesized derivatives are able to induce DNA interstrand cross-linking in colon cancer HCT-116 cells. The HCT-116 cells were treated with compound **18b** for 1 h at various concentrations under aerobic conditions and were then irradiated with X-ray radiation at a dose of 20 Gy to induce DNA strand breaks. The irradiated cells were

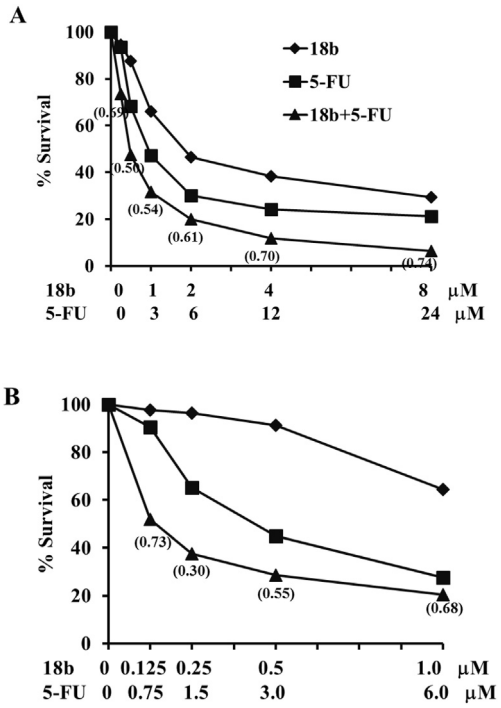


Fig. 3. Enhancement of the cytotoxicity of compound **18b** by 5-FU in the HCT-116 colon cancer cell line. Tumor cells were treated with various concentrations of compound **18b**, 5-FU, and **18b**+5-FU. (A) Combination of compound **18b** and 5-FU in a ratio of 1:3; (B) combination of compound **18b** and 5-FU in a ratio of 1:6.

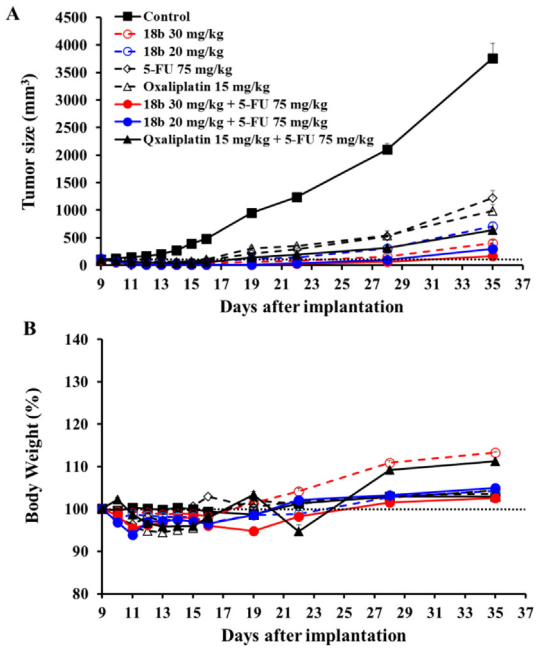


Fig. 4. Therapeutic effect of the combination of compound **18b** with 5-FU in nude mice bearing colon cancer HCT-116 xenografts. (A) Average tumor size changes; (B) average body weight changes.

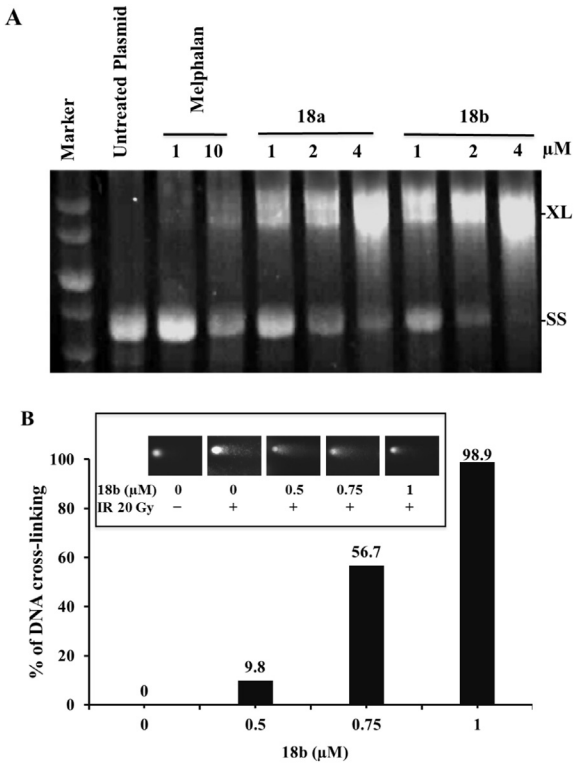


Fig. 5. DNA cross-linking induced by compound **18b**. (A) Representative DNA cross-linking gel shift assay of the newly synthesized water-soluble phenyl *N*-mustard-benzenealkylamide derivatives **18a** and **18b** at concentrations of 1, 2, and 4 μM. XL: cross-linking; SS: single strand; (B) intracellular DNA interstrand cross-linking determined by modified comet assay in colon cancer HCT-116 cells. Cells were treated with various concentrations (0.5, 0.75, and 1 μM) of compound **18b** for 1 h prior to 20-Gy X-ray irradiation.

subjected to a modified comet assay. Because irradiation causes DNA fragmentation in cells treated with DNA cross-linking agents, DNA fragments would display a reduced migration rate under alkaline electrophoresis conditions. The length and fragment content of the tail is inversely proportional to the amount of DNA interstrand cross-linking [20]. Fig. 5B shows the distribution of tail moment of HCT-116 cells after treatment with compound **18b** at concentrations of 0.5, 0.75, and 1 μM. The results demonstrate that compound **18b** can induce strong DNA interstrand cross-linking in colon carcinoma HCT-116 cells.

2.2.5. Cell cycle inhibition

We investigated the effects of compound **18b** on cell cycle progression in colon cancer HCT-116 cells (Fig. 6). HCT-116 cells were treated with compound **18b** at concentrations of 0.25, 0.5, 1, and 2 μM for 24, 48, and 72 h, followed by cell cycle distribution analysis by flow cytometry. As shown in Fig. 6, compound **18b** resulted in dose-dependent accumulation of G2/M phase cells after treatment for 24 h. However, cell cycle progression in compound **18b**-treated cells recovered after incubation for 48 h, with the exception of the 2 μM dose, which significantly delayed the progress of cell cycling (Fig. 6). The second accumulation of the G2/M phase cells as well as sub-G1 cells in HCT-116 cells treated with compound **18b** for 72 h indicated that the damage caused by this compound was irreparable and triggered apoptotic cell death.

2.2.6. Induction of apoptosis by compound *18b*

To confirm whether compound **18b** causes apoptotic cell death, we performed Western blotting to examine the appearance of active forms of caspase-3 and poly ADP-ribose polymerase (PARP), indicator proteins of apoptosis [21,22] in HCT-116 cells treated with compounds **18b** and oxaliplatin. As shown in Fig. 7A, compound **18b** induced cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). These results support that the damage induced by the compound **18b** could trigger apoptosis pathways [23]. Furthermore, we performed the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to examine the apoptotic cells in tumor tissues from mice 24 h after the completion of treatment. As shown in Fig. 7B, apoptotic signals (dark brown) were significantly increased in tumor sections from mice treated with either compound **18b** or oxaliplatin. No apoptotic signal was observed in tumors from untreated mice. These results indicate that compound **18b** can induce apoptotic cell death in xenograft tumor models.

2.2.7. Pharmacokinetic profile of compound *18b* in rats

Prior to clinical studies, we investigated the whole blood pharmacokinetics (PK) of compound **18b** in healthy male Sprague Dawley rats following either a single iv injection or an oral administration at a dose of 5.0 mg/kg or 50.0 mg/kg, respectively. Serial blood samples were collected, and the concentrations of compound **18b** in rat blood were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The system clearance of compound **18b** was rapid, and the compound was well distributed into tissues. The mean terminal half-life ($t_{1/2}$) of compound **18b** was 0.36 h and 1.01 h when administered via iv and oral administration, respectively. However, the oral bioavailability of compound **18b** was 5.32%, suggesting that this compound should be administered via iv injection. The full PK parameters of compound **18b** following an iv or oral dose to rats are given in Table S2.

2.2.8. Acute intravenous injection toxicity of compound *18b* in mice

We evaluated the 14-day acute toxicity of compound **18b** in mice via a single administration by iv injection. Compound **18b** was

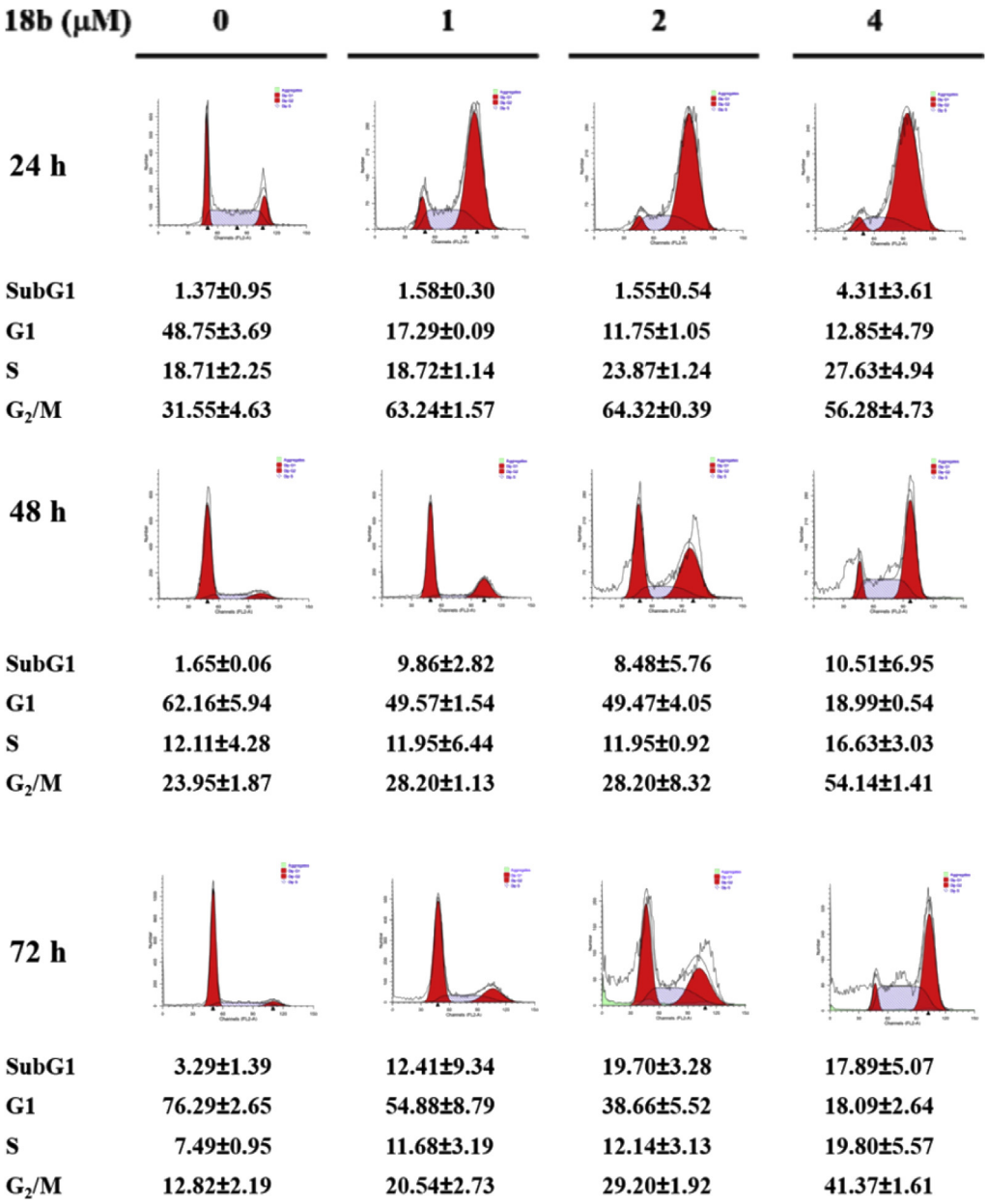


Fig. 6. Cell cycle inhibition in human colon cancer HCT-116 cells by treatment with compound **18b**.

given via a single tail vein iv injection at doses of 55, 75, 105, and 150 mg/kg to adult ICR male mice (5/group). All animals were observed for a 14-day study period after single administration of the test compound. Test compound-related changes in clinical observations, body weight, and food consumption were identified. Piloerection (goose bumps) was observed in animals at treatments of 55 mg/kg or higher. Hypoactivity was observed in animals at treatments of 75 mg/kg or higher. Swelling of the cheek, mouth, or nose, hunch posture, soft feces, feces stain, and dehydration were observed in animals treated with 150 mg/kg. These clinical signs were dose-dependent. Weight loss as well as decreased food consumption were observed in animals in the 75 and 105 mg/kg groups on D8, with recovery on D15. Test compound-related gross lesions in the spleen and thymus were observed in all of the highest-dosed animals. Under the conditions of the study, administration of compound **18b** at 150 mg/kg would result in animal death. Based on the data from the study, the LD_{50} of compound **18b** was calculated as 115.4 mg/kg. The complete data set from the acute iv injection toxicity study of compound **18b** in mice is provided in Table S3.

In addition, we examined the cardiac safety of compound **18b** using a human ether-a-go-go-related gene (hERG) fluorescence polarization assay [24]. Inhibition of the hERG ion channel by small molecule drugs can result in a long QT interval and lead to arrhythmias [25,26]. The hERG-specific binding affinity of compound **18b** was 4.48 ± 1.64 μM (from triplicate measurements), indicating that compound **18b** is less likely to display a cardiac arrhythmic side effect. The concentration–response curves for compound **18b** are provided in Fig. S5.

3. Conclusion

Our recent studies on the research and development of new stable, water-soluble antitumor N-mustard derivatives identified

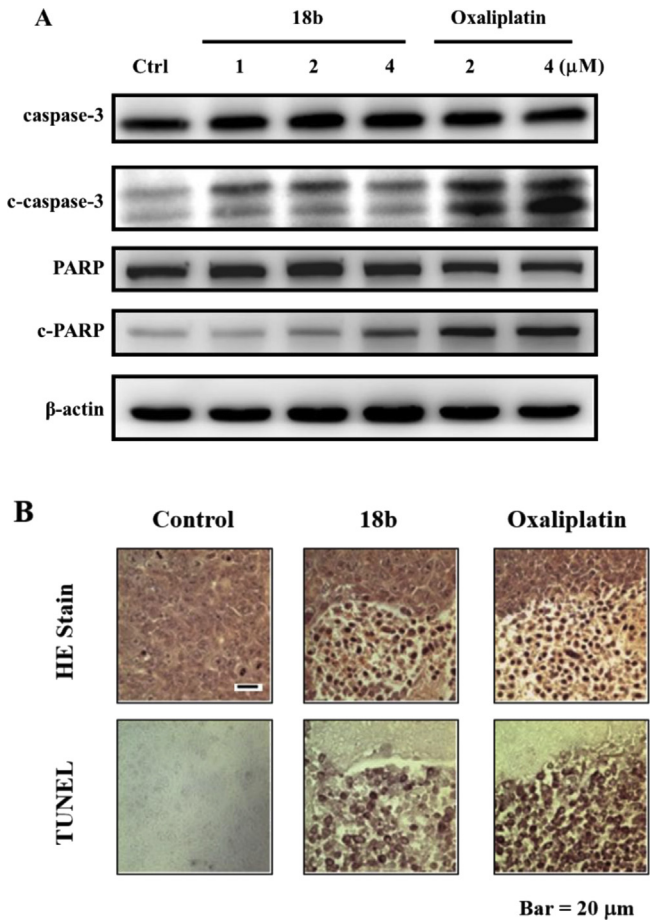


Fig. 7. Triggering of apoptotic cell death by compound **18b**. (A) Effects of compound **18b** and oxaliplatin on the levels of the apoptotic proteins caspase-3 and PARP and their cleaved forms in HCT-116 cells as assessed by Western blot analysis; (B) TUNEL assay. Xenograft tumor sections were removed from each group 1 day after the last treatment, sectioned, and subjected to TUNEL assay and HE staining. The apoptosis signal (dark brown) was significantly increased in tumor sections after treatment with compound **18b** or oxaliplatin compared to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compound **8** as a lead compound for further new antitumor drug development. Through lead optimization processes, we have synthesized a series of new water-soluble phenyl N-mustard-benzenealkylamide conjugates, which were designed via a bioisostere approach. The newly synthesized derivatives were evaluated for their antitumor effects against various human tumor cell lines. Compound **8** was used for comparison to verify the success of the lead optimization. We demonstrated that the newly synthesized compounds have a broad spectrum of antitumor activity against a panel of human tumor cell lines. Among these derivatives, compound **18b** was selected for further antitumor evaluation. Compound **18b** was more potent than compound **8** in nude mice bearing colon cancer HCT-116 and lung cancer H460 xenografts. Furthermore, we demonstrated that the therapeutic effects of compound **18b** in combination with 5-FU in HCT-116 xenograft mice were superior to those of oxaliplatin+5-FU, 5-FU alone, or oxaliplatin alone. This indicates that the combination of compound **18b**+5-FU may have high potential benefit for the treatment of advanced colon cancer. Early preclinical studies indicate that compound **18b** is likely to have no cardiac arrhythmic side effect and is less toxic than compound **8** based on a 14-day acute iv injection study, although compound **18b** has a shorter half-life in rats. In conclusion, we successfully identified a new anticancer candidate compound **18b** for preclinical studies via lead optimization development.

4. Experimental protocols

4.1. Material and methods

All commercial chemicals and solvents were reagent grade and were used without further purification unless otherwise specified. Melting points were determined in open capillaries on a Fargo MP–2D melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel G60 F254 (Merck, Merck KGaA, Darmstadt, Germany) with short-wavelength ultraviolet (UV) light for visualization. High-performance liquid chromatography (HPLC) was performed on an Elite Lachrom instrument with a Mightysil RP-18 (250 × 4.6 μm) column. Compounds were detected by UV at 260 nm. The mobile phase was acetonitrile/THF (80:20 v/v) with a flow rate of 1 mL/min. The purity of all tested compounds was ≥95% based on analytical HPLC. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on Bruker AVANCE Top-Spin spectrometers (400 and 500 MHz) in the solvents indicated. Proton chemical shifts are reported in parts per million (δ ppm) relative to $(CH_3)_4Si$ (TMS), and coupling constants (J) are reported in Hertz (Hz). NMR peak splittings are given by the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet; and brs, broad singlet. High resolution mass spectra (HRMS) were recorded on a Waters HDMS G1 instrument with ESI+, centroid mode, and the samples were dissolved in MeOH.

4.2. Chemistry

4.2.1. Compounds prepared by Method 1

Most of compounds **12**, **14**, and **15** are known and have been published elsewhere (for detailed experimental procedures for these intermediates, see the Supplementary material). We describe here the chemical syntheses of the newly synthesized compounds, starting from compound **18**.

*4.2.1.1. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(dimethylamino)-acetamide (**18a**).* To a stirred solution of **15f** (1.7 g, 8.7 mmol) in dry DMF (25 mL) containing TEA (1.6 mL) was added a solution of *N*,*N*-bis(2-chloroethyl)-4-isocyanatoaniline **17** [freshly prepared from *N*,*N*-bis-(2-chloroethyl)benzene-1,4-diamine hydrochloride **16** (5.3 g, 17 mmol) and triphosgene in chloroform] in dry DMF (10 mL) at rt. After stirring overnight at rt, the solid was filtered and washed with dry DMF. The filtrate was evaporated to dryness in vacuo, and the product was purified by column chromatography using $CHCl_3$/MeOH (100:10 v/v) as an eluant. The fractions containing the main product were combined and evaporated to dryness under vacuo to afford compound **18a**, yield: 1.9 g; ^{1}H NMR (DMSO-d_6): δ = 2.87 (s, 6H, CH_3), 3.66–3.72 (m, 8H, CH_2), 4.12 (s, 2H, CH_2), 6.71–6.73 (d, J = 8.8 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.8 Hz, 2H, Ar–H), 7.41–7.43 (d, J = 8.9 Hz, 2H, Ar–H), 7.49–7.52 (d, J = 9.0 Hz, 2H, Ar–H), 8.86 (brs, 1H, NH), 9.12 (brs, 1H, NH), 9.98 (brs, 1H, NH), 10.72 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 41.01, 43.14, 52.63, 57.72, 113.04, 117.99, 120.05, 120.08, 130.65, 131.63, 136.47, 140.99, 152.86, 162.23; HRMS [ES$^+$]: calcd for $C_{21}H_{27}Cl_2N_5O_2$, 452.3774 $[M + H]^+$, found 452.1615.

Following the same synthetic procedure as that used for **18a**, the following compounds were prepared:

*4.2.1.2. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(diethylamino)acetamide (**18b**).* Compound **18b** was prepared from **15g** (1.2 g, 5.5 mmol) and N-mustard isocyanate **17** [freshly

prepared from **16** hydrochloride (3.3 g, 11 mmol)]; Yield: 1.4 g; ^{1}H NMR (DMSO-d_6): δ = 1.23–1.27 (t, J = 7.2 Hz, 6H, CH_3), 3.21–3.25 (q, J = 7.5 Hz, 4H, CH_2), 3.68–3.71 (m, 8H, CH_2), 4.11 (s, 2H, CH_2), 6.69–6.72 (d, J = 8.6 Hz, 2H, Ar–H), 7.26–7.29 (d, J = 8.8 Hz, 2H, Ar–H), 7.41–7.43 (d, J = 8.9 Hz, 2H, Ar–H), 7.49–7.52 (d, J = 8.9 Hz, 2H, Ar–H), 8.72 (brs, 1H, NH), 8.96 (brs, 1H, NH), 9.69 (brs, 1H, NH), 10.72 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 9.37, 41.57, 48.90, 53.22, 53.31, 113.65, 118.53, 120.62, 132.24, 137.10, 153.43, 163.16; HRMS [ES$^+$]: calcd for $C_{23}H_{31}Cl_2N_5O_2$, 480.4305 [M + H]$^+$, found 480.1928.

*4.2.1.3. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(pyrrolidin-1-yl)-acetamide (**18c**).* Compound **18c** was prepared from **15h** (1.9 g, 8.6 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (5.3 g, 17 mmol)]; Yield: 1.6 g; ^{1}H NMR (DMSO-d_6): δ = 1.90–1.95 (m, 4H, CH_2), 3.11–3.17 (m, 2H, CH_2), 3.60–3.62 (m, 2H, CH_2), 3.65–3.76 (m, 8H, CH_2), 4.21–4.22 (m, 2H, CH_2), 6.71–6.73 (d, J = 8.9 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.9 Hz, 2H, Ar–H), 7.40–7.42 (d, J = 8.9 Hz, 2H, Ar–H), 7.49–7.52 (d, J = 8.9 Hz, 2H, Ar–H), 8.89 (brs, 1H, NH), 9.14 (brs, 1H, NH), 10.31 (brs, 1H, NH), 10.67 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 23.24, 41.67, 53.12, 54.49, 56.09, 113.42, 118.55, 120.53, 120.64, 131.05, 132.31, 136.96, 141.76, 153.44, 163.33; HRMS [ES$^+$]: calcd for $C_{23}H_{29}Cl_2N_5O_2$, 478.4147 [M + H]$^+$, found 478.1771.

*4.2.1.4. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(piperidin-1-yl)acetamide (**18d**).* Compound **18d** was prepared from **15i** (2.0 g, 8.6 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (5.3 g, 17 mmol)] in dry DMF. Yield: 1.9 g; ^{1}H NMR (DMSO-d_6) δ: 1.40–1.41 (m, 1H, CH), 1.68–1.79 (m, 6H, CH_2), 3.07–3.08 (m, 2H, CH_2), 3.46–3.49 (m, 2H, CH_2), 3.68–3.72 (m, 8H, CH_2), 4.08 (s, 2H, CH_2), 6.70–6.72 (d, 2H, J = 9.0 Hz, Ar–H), 7.27–7.29 (d, 2H, J = 8.9 Hz, Ar–H), 7.41–7.43 (d, 2H, J = 8.9 Hz, Ar–H), 7.50–7.52 (d, 2H, J = 8.9 Hz, Ar–H), 8.79 (brs, 1H, NH), 9.05 (brs, 1H, NH), 9.85 (1H, brs, NH), 10.70 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 21.55, 22.66, 41.69, 53.05, 53.41, 57.48, 113.30, 118.57, 120.64, 120.70, 130.87, 132.19, 137.07, 141.91, 153.42, 162.70. HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_2$, 492.4412 [M + H]$^+$, found 492.1928.

*4.2.1.5. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-morpholinoacetamide (**18e**).* Compound **18e** was prepared from **15j** (1.9 g, 8.1 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (5.0 g, 16 mmol)]; Yield: 3.13 g; ^{1}H NMR (DMSO-d_6): δ = 3.16–3.28 (m, 2H, CH_2), 3.43–3.49 (m, 2H, CH_2), 3.68–3.72 (m, 8H, CH_2), 3.79–3.85 (m, 2H, CH_2), 3.96–4.03 (m, 2H, CH_2), 4.19 (s, 2H, CH_2), 6.71–6.73 (d, J = 8.8 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.9 Hz, 2H, Ar–H), 7.41–7.43 (d, J = 8.9 Hz, 2H, Ar–H), 7.50–7.52 (d, J = 90 Hz, 2H, Ar–H), 8.87 (1H, brs, NH), 9.13 (1H, brs, NH), 10.63 (1H, brs, NH), 10.75 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 41.61, 51.70, 52.26, 53.16, 57.34, 63.51, 113.52, 118.56, 120.64, 120.67, 131.13, 132.15, 137.10, 141.66, 153.43, 162.47; HRMS [ES$^+$]: calcd for $C_{23}H_{29}Cl_2N_5O_3$, 494.4141 [M + H]$^+$, found 494.1720.

*4.2.1.6. 2-([1,4′-Bipiperidin]-1′-yl)-N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-acetamide (**18f**).* Compound **18f** was prepared from **15k** (1.9 g, 6.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.7 g, 12 mmol)]; Yield: 2.2 g; ^{1}H NMR (DMSO-d_6): δ = 1.40–1.43 (m, 1H, CH), 1.69–1.72 (m, 1H, CH), 1.79–1.92 (m, 4H, CH_2), 2.16–2.22 (m, 2H, CH_2), 2.33–2.36 (m, 2H, CH_2), 2.88–2.96 (m, 2H, CH_2), 3.22–3.25 (m, 2H, CH_2), 3.36–3.39 (m, 3H, CH), 3.66–3.73 (m, 10H, CH_2), 4.11–4.15 (m, 2H, CH_2), 6.69–6.72 (d, J = 9.0 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 9.0 Hz, 2H, Ar–H), 7.41–7.43 (m, J = 8.9 Hz, 2H, Ar–H), 7.50–7.52 (m, J = 8.9 Hz, 2H, Ar–H), 8.82 (1H, brs, NH), 9.09 (1H, brs, NH), 10.35 (1H, brs, NH), 10.75 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 21.43, 22.33, 23.05, 41.24, 49.01, 50.93, 52.57, 56.70, 59.07, 112.83, 118.02, 118.27, 120.16, 131.68, 136.64, 152.98, 162.04; HRMS [ES$^+$]: calcd for $C_{29}H_{40}Cl_2N_6O_2$, 575.5729 [M + H]$^+$, found 575.2663.

*4.2.1.7. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)-phenyl)-3-(dimethylamino)-propanamide (**19a**).* Compound **19a** was prepared from **15l** (1.15 g, 5.5 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.4 g, 11 mmol)]; Yield: 1.6 g; ^{1}H NMR (DMSO-d_6): δ = 2.77 (s, 6H, CH_3), 2.84–2.87 (t, J = 7.1 Hz, 2H, CH_2), 3.32–3.35 (t, J = 6.7 Hz, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.71–6.73 (d, J = 8.6 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.8 Hz, 2H, Ar–H), 7.36–7.38 (d, J = 8.8 Hz, 2H, Ar–H), 7.48–7.50 (d, J = 8.8 Hz, 2H, Ar–H), 7.95 (1H, brs, NH), 8.82 (1H, brs, NH), 9.02 (1H, brs, NH), 10.24 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 30.50, 41.00, 42.10, 52.52, 52.58, 112.95, 117.95, 119.74, 120.02, 130.64, 132.64, 135.77, 140.96, 152.85, 167.25; HRMS [ES$^+$]: calcd for $C_{22}H_{29}Cl_2N_5O_2$, 466.4040 [M + H]$^+$, found 466.1771.

*4.2.1.8. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-(diethylamino)-propanamide (**19b**).* Compound **19b** was prepared from **15m** (1.2 g, 5.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.0 g, 10 mmol)]; Yield: 1.6 g; ^{1}H NMR (DMSO-d_6): δ = 1.22–1.25 (t, J = 7.1 Hz, 6H, CH_3), 2.85–2.88 (t, J = 7.1 Hz, 2H, CH_2), 3.11–3.14 (m, 4H, CH_2), 3.33–3.34 (m, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.73–6.75 (d, J = 8.2 Hz, 2H, Ar–H), 7.28–7.30 (d, J = 8.6 Hz, 2H, Ar–H), 7.36–7.38 (d, J = 8.7 Hz, 2H, Ar–H), 7.49–7.51 (d, J = 8.8 Hz, 2H, Ar–H), 8.90 (brs, 1H, NH), 9.09 (brs, 1H, NH), 10.26 (brs, 1H, NH), 10.30 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 8.35, 30.11, 41.07, 46.32, 46.72, 52.49, 112.76, 118.0, 119.71, 120.09, 130.39, 132.69, 135.74, 141.19, 152.83, 167.22; HRMS [ES$^+$]: calcd for $C_{24}H_{33}Cl_2N_5O_2$, 494.4571 [M + H]$^+$, found 494.2078.

*4.2.1.9. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-(pyrrolidin-1-yl)-propanamide (**19c**).* Compound **19c** was prepared from **15n** (1.2 g, 5.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.0 g, 10 mmol)]; Yield: 1.2 g; ^{1}H NMR (DMSO-d_6) δ: 1.88–1.99 (m, 4H, CH_2), 2.83–2.87 (t, J = 7.1 Hz, 2H, CH_2), 3.02–3.10 (m, 2H, CH_2), 3.39–3.41 (m, 2H, CH_2), 3.49–3.51 (m, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.69–6.71 (d, J = 8.8 Hz, 2H, Ar–H), 7.26–7.28 (d, J = 8.8 Hz, 2H, Ar–H), 7.36–7.38 (d, J = 8.8 Hz, 2H, Ar–H), 7.48–7.50 (d, J = 8.8 Hz, 2H, Ar–H), 8.70 (brs, 1H, NH), 8.91 (brs, 1H, NH), 10.20 (brs, 1H, NH), 10.47 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 22.59, 31.80, 41.03, 49.70, 52.54, 52.91, 112.87, 117.99, 119.73, 120.08, 130.53, 132.69, 135.73, 141.08, 152.84, 167.12; HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_2$, 492.4412 [M + H]$^+$, found 492.1928.

*4.2.1.10. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-(piperidin-1-yl)-propanamide (**19d**).* Compound **19d** was prepared from **15o** (1.2 g, 5.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.0 g, 10 mmol)]; Yield: 1.8 g; ^{1}H NMR (DMSO-d_6): δ = 1.33–1.42 (m, 1H, CH), 1.68–1.79 (m, 5H, CH), 2.86–2.89 (m, 4H, CH_2), 3.30–3.35 (m, 2H, CH_2), 3.39–3.42 (m, 2H, CH_2), 3.68–3.72 (m, 8H, CH_2), 6.70–6.72 (d, J = 8.8 Hz, 2H, Ar–H), 7.26–7.28 (d, J = 8.8 Hz, 2H, Ar–H), 7.35–7.38 (d, J = 8.8 Hz, 2H, Ar–H), 7.47–7.49 (d, J = 8.8 Hz, 2H, Ar–H), 8.66 (brs, 1H, NH), 8.86 (brs, 1H, NH), 10.04 (brs, 1H, NH) 10.18 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 21.17, 22.25, 30.20, 41.10, 51.70, 52.01, 52.44, 112.66, 118.02, 119.74, 120.15, 130.26, 132.71, 135.72, 141.31, 152.82, 167.21; HRMS [ES$^+$]: calcd for $C_{25}H_{33}Cl_2N_5O_2$, 506.4678 [M + H]$^+$, found 506.2084.

*4.2.1.11. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-morpholinopropanamide (**19e**).* Compound **19e** was prepared from **15p** (1.3 g, 5.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.0 g, 10 mmol)]; Yield: 1.7 g; ^{1}H NMR

(DMSO-d_6): δ = 2.89–2.91 (m, 2H, CH_2), 3.08–3.17 (m, 6H, CH_2), 3.68–3.70 (m, 8H, CH_2), 3.77–3.79 (m, 2H, CH_2), 3.94–3.96 (m, 2H, CH_2), 6.69–6.71 (d, J = 7.4 Hz, 2H, Ar–H), 7.26–7.28 (d, J = 7.4 Hz, 2H, Ar–H), 7.36–7.39 (m, 2H, Ar–H), 7.47–7.49 (m, 2H, Ar–H), 8.70 (brs, 1H, NH), 8.91 (brs, 1H, NH), 10.21 (brs, 1H, NH), 10.89 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 29.83, 41.05, 51.01, 51.82, 52.52, 63.04, 112.82, 117.99, 119.76, 120.10, 130.45, 132.68, 135.74, 141.11, 152.83, 167.04; HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_3$, 508.4406 [M + H]$^+$, found 508.1877.

*4.2.1.12. 3-([1,4′-Bipiperidin]-1′-yl)-N-(4-(3-(4-(bis(2-chloroethyl) amino)phenyl)ureido)phenyl) propanamide (**19f**).* Compound **19f** was prepared from **15q** (2.2 g, 5.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (4.08 g, 13 mmol)]; Yield: 1.9 g; ^{1}H NMR (DMSO-d_6): δ = 1.39–1.42 (m, 1H, CH), 1.67–1.70 (m, 1H, CH), 1.78–1.90 (m, 4H, CH_2), 2.11–2.20 (m, 2H, CH_2), 2.33–2.36 (m, 2H, CH_2), 3.60–3.73 (m, 9H, CH_2), 6.71–6.73 (d, J = 8.9 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.9 Hz, 2H, Ar–H), 7.36–7.38 (m, J = 8.9 Hz, 2H, Ar–H), 7.48–7.50 (m, J = 8.9 Hz, 2H, Ar–H), 8.83 (brs, 1H, NH), 9.03 (brs, 1H, NH), 10.24 (brs, 1H, NH), 10.78 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 21.44, 22.15, 22.40, 23.11, 30.42, 41.03, 49.03, 50.24, 51.68, 52.90, 59.34, 113.51, 118.07, 119.92, 120.06, 132.84, 135.88, 153.01, 167.19; HRMS [ES$^+$]: calcd for $C_{30}H_{42}Cl_2N_6O_2$, 589.5995 [M + H]$^+$, found 589.2819.

*4.2.1.13. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-3-(dimethylamino)propanamide (**21a**).* Compound **21a** was prepared from **15a** (1.15 g, 5.5 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.4 g, 11 mmol)]; Yield: 2.2 g; ^{1}H NMR (DMSO-d_6): δ = 2.77 (s, 6H, CH_3), 2.87–2.90 (t, J = 7.1 Hz, 2H, CH_2), 3.33–3.36 (t, J = 6.7 Hz, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.74–6.76 (d, J = 8.6 Hz, 2H, Ar–H), 7.27–7.29 (d, J = 8.8 Hz, Ar 2H, –H), 7.36–7.38 (d, J = 8.8 Hz, 2H, Ar–H), 7.48–7.50 (d, J = 8.8 Hz, 2H, Ar–H), 7.95 (brs, 1H, NH), 8.82 (brs, 1H, NH), 9.02 (brs, 1H, NH), 10.37 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 30.90, 40.81, 42.30, 52.66, 53.32, 79.35, 108.86, 112.65, 113.14, 114.30, 120.00, 128.93, 139.27, 140.51, 152.90, 167.84; HRMS [ES$^+$]: calcd for $C_{22}H_{29}Cl_2N_5O_2$, 466.4040 [M + H]$^+$, found 466.1771.

*4.2.1.14. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-3-(diethylamino)propanamide (**21b**).* Compound **21b** was prepared from **15b** (3.1 g, 12.75 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (7.8 g, 25.5 mmol)]; Yield: 4.0 g; ^{1}H NMR (DMSO-d_6): δ = 1.23–1.26 (t, J = 7.1 Hz, 6H, CH_3), 2.87–2.91 (t, J = 7.1 Hz, 2H, CH_2), 3.11–3.14 (m, 4H, CH_2), 3.34–3.35 (m, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.75–6.77 (d, J = 8.3 Hz, 2H, Ar–H), 7.16–7.17 (m, 2H, Ar–H), 7.28–7.32 (m, 3H, Ar–H), 7.73 (s, 1H, Ar–H), 9.04 (brs, 1H, NH), 9.21 (brs, 1H, NH), 10.31 (brs, 1H, NH), 10.34 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 8.51, 30.42, 40.96, 46.48, 46.76, 53.02, 108.74, 112.52, 113.02, 113.67, 120.0, 128.88, 139.28, 140.52, 152.87, 167.79. HRMS [ES$^+$]: calcd for $C_{24}H_{33}Cl_2N_5O_2$, 494.4571 [M + H]$^+$, found 494.2090.

*4.2.1.15. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-3-(pyrrolidin-1-yl)propanamide (**21c**).* Compound **21c** was prepared from **15c** (2.3 g, 10.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (6.1 g, 20 mmol)]; Yield: 3.5 g; ^{1}H NMR (DMSO-d_6): δ = 1.87–1.90 (m, 2H, CH_2), 1.99–2.01 (m, 2H, CH_2), 2.89–2.92 (t, J = 6.0 Hz, 2H, CH_2), 3.02–3.05 (m, 2H, CH_2), 3.40–3.44 (m, 2H, CH_2), 3.47–3.51 (m, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 6.81–6.83 (d, J = 6.4 Hz, 2H, Ar–H), 7.15–7.19 (m, 2H, Ar–H), 7.27–7.29 (m, 1H, Ar–H), 7.33–7.35 (d, J = 7.1 Hz, 2H, Ar–H), 7.75 (s, 1H, Ar–H), 9.16 (brs, 1H, NH), 9.29 (brs, 1H, NH), 10.33 (brs, 1H, NH), 10.84 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 22.75, 32.14, 40.96, 49.76, 53.01, 56.04, 108.75, 112.53, 113.02, 113.71, 120.01, 128.88, 139.28, 140.51, 152.87, 167.68; HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_2$, 492.4412 [M + H]$^+$, found 492.1933.

*4.2.1.16. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-3-(piperidin-1-yl)propanamide (**21d**).* Compound **21d** was prepared from **15d** (2.47 g, 10.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (6.1 g, 20 mmol)]; Yield: 4.2 g; ^{1}H NMR (DMSO-d_6): δ = 1.33–1.42 (m, 1H, CH), 1.67–1.79 (m, 5H, CH), 2.90–2.94 (m, 4H, CH_2), 3.30–3.33 (m, 2H, CH_2), 3.38–3.42 (m, 2H, CH_2), 3.69–3.74 (m, 8H, CH_2), 6.73–6.75 (m, 2H, Ar–H), 7.16–7.18 (m, 2H, Ar–H), 7.26–7.27 (m, 3H, Ar–H), 7.72 (s, 1H, Ar–H), 8.95 (brs, 1H, NH), 9.14 (brs, 1H, NH), 10.30 (brs, 1H, NH), 10.32 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 21.32, 22.37, 30.53, 40.89, 51.79, 52.13, 53.14, 108.79, 112.57, 113.03, 113.97, 119.99, 128.88, 139.31, 140.50, 152.87, 167.79; HRMS [ES$^+$]: calcd for $C_{25}H_{33}Cl_2N_5O_2$, 506.4678 [M + H]$^+$, found 506.2090. HPLC 98.8%.

*4.2.1.17. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-3-morpholinopropanamide (**21e**).* Compound **21e** was prepared from **15e** (1.5 g, 6.0 mmol) and N-mustard isocyanate **17** [freshly prepared from **16** (3.67 g, 12 mmol)]; Yield: 1.6 g; ^{1}H NMR (DMSO-d_6): δ = 2.95–2.98 (m, 2H, CH_2), 3.11–3.13 (m, 2H, CH_2), 3.39–3.42 (m, 4H, CH_2), 3.68–3.70 (m, 8H, CH_2), 3.81–3.86 (m, 2H, CH_2), 3.95–3.98 (m, 2H, CH_2), 6.79–6.81 (m, 2H, Ar–H), 7.16–7.18 (m, 2H, Ar–H), 7.28–7.34 (m, 3H, Ar–H), 7.75 (s, 1H, Ar–H), 9.15 (brs, 1H, NH), 9.29 (brs, 1H, NH), 10.32 (brs, 1H, NH), 11.31 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 30.17, 41.02, 51.14, 51.89, 52.95, 63.17, 108.77, 112.54, 113.03, 120.03, 121.19, 128.87, 139.26, 140.51, 152.87, 167.61; HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_3$, 508.4406 [M + H]$^+$, found 508.1882.

4.2.2. Compounds prepared by Method 2

*4.2.2.1. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(3-nitrophenyl) urea (**23a**).* To a stirred mixture of *N*-mustard amine **16** (7.66 g, 25 mmol) and TEA (4 mL, 27.5 mmol) in $CHCl_3$ (100 mL) was added dropwise a solution of 3-nitrophenyl isocyanate **22a** (4.1 g, 25 mmol) in $CHCl_3$ (100 mL) at rt. After stirring for an additional 40–45 min, the solvent was removed by evaporation under reduced pressure, and the solid residue was triturated with a saturated aqueous solution of $NaHCO_3$ and collected by filtration. The solid was washed successively with water and hexane, followed by drying. The solid was recrystallized from ethyl acetate to give **23a**. Yield: 9.1 g (92%); mp: 246–247 °C; ^{1}H NMR (DMSO-d_6): δ = 3.69–3.71 (m, 8H, CH_2), 6.71–6.74 (d, J = 9.0 Hz, 2H, Ar–H), 7.29–7.31 (d, J = 8.9 Hz, 2H, Ar–H), 7.52–7.56 (t, J = 8.1 and 8.2 Hz, 1H, Ar–H), 7.69–7.71 (d, J = 8.0 Hz, 1H, Ar–H), 7.78–7.80 (q, J = 8.2 and 1.8 Hz, 1H, Ar–H), 8.52 (brs, 1H, NH), 8.54–8.55 (d, J = 2 Hz, 1H, Ar–H), 9.11 (brs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.23, 52.39, 111.85, 112.46, 115.85, 121.06, 124.05, 129.28, 129.95, 141.42, 142.18, 148.14, 152.65; HRMS [ES$^+$]: calcd for $C_{17}H_{18}Cl_2N_4O_3$, 397.0834 [M + H]$^+$, found 397.0833.

Following the same synthetic procedure as that used for **23a**, the following compound was prepared:

*4.2.2.2. 1-(4-(Bis(2-chloroethyl)amino)phenyl)-3-(4-nitrophenyl) urea (**23b**).* Compound **23b** was prepared from **22b** (4.1 g, 25.0 mmol), *N*-mustard amine **16** (7.6 g, 25.0 mmol), and TEA (4 mL, 27.6 mmol); Yield: 8.95 g (90%); mp: 181–183 °C; ^{1}H NMR (DMSO-d_6): δ = 3.68–3.70 (m, 8H, CH_2), 6.71–6.73 (d, J = 8.6 Hz, 2H, Ar–H), 7.30–7.32 (d, J = 8.5 Hz, 2H, Ar–H), 7.66–7.68 (d, J = 8.7 Hz, 2H, Ar–H), 8.14–8.17 (d, J = 8.6 Hz, 2H, Ar–H), 9.11 (brs, 2H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.25, 52.36, 112.40, 117.19, 121.05, 125.20, 129.07, 140.64, 142.30, 146.87, 152.18; HRMS [ES$^+$]: calcd for $C_{17}H_{18}Cl_2N_4O_3$, 397.0834 [M + H]$^+$, found 397.0832.

*4.2.2.3. 1-(3-Aminophenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl) urea (**24a**).* A solution of compound **23a** (3.5 g, 8.8 mmol) in EA (100 mL) was hydrogenated with H_2 gas in a Parr hydrogenator using 10% Pd/C (0.8 g) as a catalyst at 30–35 psi for 6–7 h. After completion of the reaction, the mixture was filtered through a Celite pad, and the filtrate was evaporated to dryness under reduced pressure to afford **24a**. Yield: 3.0 g (93%); mp: 182–183 °C; ^{1}H NMR (DMSO-d_6): δ = 3.66–3.72 (m, 8H, CH_2), 5.22 (brs, 2H, NH_2), 6.17–6.19 (d, J = 8.0 Hz, 1H, Ar–H), 6.54–6.56 (d, J = 7.9 Hz, 1H, Ar–H), 6.69–6.71 (d, J = 9.0 Hz, 2H, Ar–H), 6.86 (s, 1H, Ar–H), 6.88–6.90 (t, J = 8.0 Hz, 1H, Ar–H), 7.25–7.28 (d, J = 8.9 Hz, 2H, Ar–H), 8.27 (brs, 2H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.30, 52.47, 104.20, 106.76, 108.39, 112.57, 120.35, 129.10, 130.13, 140.66, 141.67, 147.86, 152.69; HRMS [ES$^+$]: calcd for $C_{17}H_{20}Cl_2N_4O$, 367.1092 [M + H]$^+$, found 367.1088.

Following the same synthetic procedure as that used for **24a**, the following compound was prepared:

*4.2.2.4. 1-(4-Aminophenyl)-3-(4-(bis(2-chloroethyl)amino)phenyl) urea (**24b**) [27].* Compound **24b** was prepared from **23b** (8.0 g, 20.0 mmol); Yield: 6.3 g (96%); mp: 198–200 °C; ^{1}H NMR (DMSO-d_6): δ = 3.66–3.70 (m, 8H, CH_2), 4.73 (brs, 2H, NH_2), 6.48–6.50 (d, J = 8.5 Hz, 2H, Ar–H), 6.67–6.70 (d, J = 8.8 Hz, 2H, Ar–H), 7.04–7.06 (d, J = 8.5 Hz, 2H, Ar–H), 7.24–7.26 (d, J = 8.8 Hz, 2H, Ar–H), 7.99 (brs, 1H, NH), 8.14 (brs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.32, 52.51, 112.61, 114.16, 120.28, 120.49, 129.0, 130.54, 141.46, 143.77, 153.26; HRMS [ES$^+$]: calcd for $C_{17}H_{20}Cl_2N_4O$, 367.1092 [M + H]$^+$, found 367.1088.

*4.2.2.5. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-chloroacetamide (**25a**).* To a solution of **24a** (3.0 g, 8.0 mmol) in THF (100 mL), chloroacetyl chloride (2.2 g, 20 mmol) in THF (50 mL) was added dropwise at rt, and the resulting mixture was stirred at rt for an additional 45 min. The reaction mixture was evaporated under reduced pressure, and the solid residue was triturated with a saturated aqueous solution of $NaHCO_3$ and filtered. The collected solid was washed successively with water and hexane and dried to yield **25a**. Yield: 2.8 g (82%); mp: 232–233 °C; ^{1}H NMR (DMSO-d_6): δ = 2.80–2.83 (t, J = 5.9 Hz, 2H, CH_2), 3.68–3.71 (m, 8H, CH_2), 3.86–3.89 (t, J = 5.9 Hz, 2H, CH_2), 6.70–6.72 (d, J = 8.5 Hz, 2H, Ar–H), 7.14–7.30 (m, 5H, Ar–H), 7.76 (s, 1H, Ar–H), 8.46 (brs, 1H, NH), 8.73 (brs, 1H, NH), 10.04 (brs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.22, 43.70, 52.62, 108.78, 112.55, 112.85, 113.41, 120.38, 129.12, 138.95, 140.56, 152.76, 164.61; HRMS [ES$^+$]: calcd for $C_{19}H_{21}Cl_3N_4O_2$, 443.0808 [M + H]$^+$, found 443.0776.

Following the same synthetic procedure as that used for **25a**, the following compounds were prepared:

*4.2.2.6. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-chloropropanamide (**25b**).* Compound **25b** was prepared from **24a** (3.0 g, 8.0 mmol) and 3-chloropropionyl chloride (2.5 g, 20 mmol); Yield: 2.8 g (82%); mp: 232–233 °C; ^{1}H NMR (DMSO-d_6): δ = 2.80–2.83 (t, J = 5.9 Hz, 2H, CH_2), 3.68–3.71 (m, 8H, CH_2), 3.86–3.89 (t, J = 5.9 Hz, 2H, CH_2), 6.70–6.72 (d, J = 8.5 Hz, 2H, Ar–H), 7.14–7.30 (m, 5H, Ar–H), 7.76 (s, 1H, Ar–H), 8.46 (brs, 1H, NH), 8.73 (brs, 1H, NH), 10.04 (brs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 40.94, 41.28, 52.44, 108.70, 112.41, 112.53, 112.98, 120.48, 128.98, 129.94, 139.39, 140.42, 141.79, 152.70, 167.93; HRMS [ES$^+$]: calcd for $C_{20}H_{23}Cl_3N_4O_2$, 457.0965 [M + H]$^+$, found 457.0960.

*4.2.2.7. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-chloroacetamide (**25c**).* Compound **25c** was prepared from **24b** (3.0 g, 8.2 mmol) and chloroacetyl chloride (1.0 g, 8.9 mmol); Yield: 3.28 g (90%); mp: 212–214 °C; ^{1}H NMR (DMSO-d_6): δ = 3.68–3.71 (m, 8H, CH_2), 4.22 (s, 2H, CH_2), 6.69–6.72 (d, J = 9.0 Hz, 2H, Ar–H), 7.26–7.28 (d, J = 8.9 Hz, 2H, Ar–H), 7.38–7.40 (d, J = 8.9 Hz, 2H, Ar–H), 7.47–7.49 (d, J = 8.9 Hz, 2H, Ar–H), 8.33 (brs, 1H, NH), 8.52 (brs, 1H, NH), 10.16 (brs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 41.30, 43.59, 52.47, 112.55, 118.32, 120.06, 120.52, 130.06, 132.41, 136.23, 141.71, 152.90, 164.17; HRMS [ES$^+$]: calcd for $C_{19}H_{21}Cl_3N_4O_2$, 443.0808 [M + H]$^+$, found 443.0807.

*4.2.2.8. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-3-chloropropanamide (**25d**).* Compound **25d** was prepared from **24b** (2.94 g, 8.0 mmol) and 3-chloropropionyl chloride (1.5 g, 12 mmol); Yield: 3.2 g (87%); mp: 213–215 °C; ^{1}H NMR (DMSO-d_6): δ = 2.77–2.80 (m, 2H, CH_2), 3.68–3.70 (m, 8H, CH_2), 3.86–3.89 (m, 2H, CH_2), 6.69–6.71 (d, 2H, Ar–H, J = 8.1 Hz), 7.26–7.28 (d, 2H, Ar–H, J = 8.2 Hz), 7.35–7.37 (d, 2H, Ar–H, J = 8.1 Hz), 7.48–7.50 (d, 2H, Ar–H, J = 8.3 Hz), 8.34 (br s, 1H, NH), 8.51 (br s, 1H, NH), 9.94 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ = 40.99, 41.29, 52.47, 112.54, 118.34, 120.55, 130.01, 133.08, 135.63, 141.75, 141.82, 152.88, 167.45; HRMS [ES$^+$]: calcd for $C_{20}H_{23}Cl_3N_4O_2$, 457.0965 [M + H]$^+$, found 457.0967.

*4.2.2.9. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(dimethylamino)acetamide hydrochloride (**20a**).* To a solution of **25a** (0.88 g, 2 mmol) in THF (50 mL) was added dropwise dimethylamine (2 M solution in THF, 2 mL, 4.0 mmol). The reaction mixture was heated at 50–60 °C overnight. After cooling the mixture, the solvent was removed by evaporation under reduced pressure. The residue obtained was triturated with a saturated aqueous solution of $NaHCO_3$. The solid product was collected by filtration, washed successively with water and hexane, and dried to yield **20a**. Yield: 0.56 g. The product was dissolved in ethanol, and excess HCl in EA was added dropwise. The solution was evaporated in vacuo to dryness, and the residue was co-evaporated several times with EtOH to form **20a** hydrochloride. ^{1}H NMR (DMSO-d_6): δ = 2.88 (s, 6H, CH_3), 3.68–3.71 (m, 8H, CH_2), 4.13 (s, 2H, CH_2), 6.71–6.73 (d, J = 8.8 Hz, 2H, Ar–H), 7.16–7.30 (m, 5H, Ar–H), 7.78 (s, 1H, Ar–H), 8.95 (brs, 1H, NH), 9.24 (brs, 1H, NH), 9.99 (brs, 1H, NH), 10.72 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 41.09, 43.25, 52.73, 57.88, 108.84, 112.56, 113.17, 113.55, 120.03, 129.04, 138.31, 140.73, 152.86, 162.91; HRMS [ES$^+$]: calcd for $C_{21}H_{27}Cl_2N_5O_2$: 452.3774 [M + H]$^+$, found 452.1620.

Following the same synthetic procedure as that used for **20a**, the following compounds were prepared:

*4.2.2.10. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(diethylamino)acetamide (**20b**).* Compound **20b** was prepared from **25a** (0.75 g, 1.7 mmol) and diethylamine (0.8 mL, 3.4 mmol); Yield: 0.66 g; ^{1}H NMR (DMSO-d_6): δ = 1.23–1.27 (t, J = 7.2 Hz, 6H, CH_3), 3.22–3.25 (q, J = 7.2 Hz, 4H, CH_2), 3.68–3.71 (m, 8H, CH_2), 4.12 (s, 2H, CH_2), 6.70–6.73 (d, J = 9.0 Hz, 2H, Ar–H), 7.15–7.30 (m, 5H, Ar–H), 7.81 (s, 1H, Ar–H), 8.87 (brs, 1H, NH), 9.17 (brs, 1H, NH), 9.71 (brs, 1H, NH), 10.76 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 8.91, 41.12, 48.49, 52.73, 52.95, 108.85, 112.54, 113.17, 113.64, 120.06, 129.07, 138.33, 140.75, 152.85, 163.18; HRMS [ES$^+$]: calcd for $C_{23}H_{31}Cl_2N_5O_2$, 480.4305 [M + H]$^+$, found 480.1933.

*4.2.2.11. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(pyrrolidin-1-yl)acetamide (**20c**).* Compound **20c** was prepared from **25a** (2.2 g, 5.0 mmol) and pyrrolidine (1.6 g, 20 mmol); Yield: 1.6 g; ^{1}H NMR (DMSO-d_6): δ = 1.90–1.93 (m, 2H, CH_2), 1.98–2.01 (m, 2H, CH_2), 3.11–3.15 (m, 2H, CH_2), 3.61–3.64 (m, 2H, CH_2), 3.66–3.72 (m, 8H, CH_2), 6.72–6.74 (d, J = 7.8 Hz, 2H, Ar–H), 7.19–7.22 (d, J = 7.6 Hz, 2H, Ar–H), 7.29–7.32 (m, 3H, Ar–H), 7.78 (s, 1H, Ar–H), 9.04 (brs, 1H, NH), 9.31 (brs, 1H, NH), 10.35 (brs, 1H, NH), 10.70 (s, 1H, HCl); ^{13}C NMR (DMSO-d_6): δ = 22.82, 41.05, 52.88, 54.04, 55.72, 108.81, 112.53, 113.50, 120.03, 129.05, 138.43,

140.72, 152.87, 163.35; HRMS [ES$^+$]: calcd for $C_{23}H_{29}Cl_2N_5O_2$, 478.4147 [M + H]$^+$, found 478.1777.

*4.2.2.12. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-2-(piperidin-1-yl)acetamide (**20d**).* Compound **20d** was prepared from **25a** (0.75 g, 1.7 mmol) and piperidine (0.4 mL, 3.4 mmol); Yield: 0.61 g; ^{1}H NMR (DMSO-d$_6$): δ = 2.92–2.96 (m, 2H, CH_2), 3.08–3.11 (m, 3H, CH), 3.38–3.41 (m, 3H, CH), 3.65–3.70 (m, 8H, CH_2), 3.78–3.84 (m, 2H, CH_2), 3.95–3.97 (m, 2H, CH_2), 6.72–6.74 (d, J = 8.7 Hz, 2H, Ar–H), 7.15–7.19 (d, J = 8.4 Hz, 2H, Ar–H), 7.26–7.32 (m, 3H, Ar–H), 7.72 (s, 1H, Ar–H), 8.94 (brs, 1H, NH), 9.14 (brs, 1H, NH), 10.29 (brs, 1H, NH), 11.14 (s, 1H, HCl); ^{13}C NMR (DMSO-d$_6$): δ = 21.09, 22.20, 41.11, 52.76, 52.93, 57.05, 108.88, 112.56, 113.18, 113.59, 120.03, 129.03, 138.33, 140.74, 152.86, 162.71; HRMS [ES$^+$]: calcd for $C_{24}H_{31}Cl_2N_5O_2$, 492.4412 [M + H]$^+$, found 492.1933.

*4.2.2.13. N-(3-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido) phenyl)-2-morpholinoacetamide (**20e**).* Compound **20e** was prepared from **25a** (0.75 g, 1.7 mmol) and morpholine (0.4 mL, 3.4 mmol); Yield: 0.59 g; ^{1}H NMR (DMSO-d$_6$): δ = 3.27–3.29 (m, 2H, CH_2), 3.46–3.50 (m, 2H, CH_2), 3.66–3.72 (m, 8H, CH_2), 3.81–3.83 (m, 2H, CH_2), 3.93–3.95 (m, 2H, CH_2), 4.20 (s, 2H, CH_2), 6.70–6.73 (d, J = 8.8 Hz, 2H, Ar–H), 7.15–7.30 (m, 5H, Ar–H), 7.80 (s, 1H, Ar–H), 8.90 (brs, 1H, NH), 9.19 (brs, 1H, NH), 10.56 (brs, 1H, NH), 10.73 (s, 1H, HCl); ^{13}C NMR (DMSO-d$_6$): δ = 41.07, 51.82, 52.82, 56.92, 63.06, 108.91, 112.60, 113.32, 113.63, 120.03, 129.07, 138.29, 140.75, 152.87, 162.50; HRMS [ES$^+$]: calcd for $C_{23}H_{29}Cl_2N_5O_3$, 494.4141 [M + H]$^+$, found 494.1726.

*4.2.2.14. 2-([1,4′-Bipiperidin]-1′-yl)-N-(3-(3-(4-(bis(2-chloroethyl) amino)phenyl)ureido)phenyl)-acetamide (**20f**).* Compound **20f** was prepared from **25a** (0.89 g, 2.0 mmol) and 4-piperidinopiperidine (0.7 g, 4.4 mmol); Yield: 0.86 g; ^{1}H NMR (DMSO-d$_6$): δ = 1.41–1.43 (m, 1H, CH), 1.69–1.81 (m, 4H, CH), 1.91–1.98 (m, 3H, CH), 2.18–2.24 (m, 2H, CH_2), 2.34–2.37 (m, 2H, CH_2), 2.91–2.93 (m, 3H, CH), 3.22–3.25 (m, 2H, CH_2), 3.35–3.38 (m, 3H, CH), 3.66–3.73 (m, 8H, CH_2), 4.15 (s, 2H, CH_2), 6.71–6.73 (d, J = 8.6 Hz, 2H, Ar–H), 7.19–7.21 (d, J = 6.5 Hz, 2H, Ar–H), 7.29–7.31 (m, 3H, Ar–H), 7.79 (s, 1H, Ar–H), 9.10 (brs, 1H, NH), 9.36 (brs, 1H, NH), 10.43 (brs, 1H, NH), 11.14 (s, 1H, HCl); ^{13}C NMR (DMSO-d$_6$): δ = 21.10, 21.46, 22.32, 23.06, 41.82, 48.99, 50.95, 52.72, 56.75, 59.08, 108.86, 112.56, 113.15, 113.59, 120.00, 129.04, 138.30, 140.75, 152.88, 162.55, 171.94; HRMS [ES$^+$]: calcd for $C_{29}H_{40}Cl_2N_6O_2$, 575.5729 [M + H]$^+$, found 575.2663.

*4.2.2.15. 3-([1,4′-Bipiperidin]-1′-yl)-N-(3-(3-(4-(bis(2-chloroethyl) amino)phenyl)- ureido)-phenyl)propanamide (**21f**).* Compound **21f** was prepared from **25b** (0.92 g, 2.0 mmol) and 4-piperidinopiperidine (0.38 g, 2.2 mmol); Yield: 1.0 g; ^{1}H NMR (DMSO-d$_6$): δ = 1.39–1.42 (m, 1H, CH), 1.68–1.71 (m, 1H, CH), 1.76–1.91 (m, 7H, CH), 1.97–1.99 (m, 1H, CH), 2.13–2.20 (m, 2H, CH_2), 2.33–2.36 (m, 2H, CH_2), 2.93–3.04 (m, 7H, CH), 3.60–3.71 (m, 10H, CH_2), 6.71–6.73 (d, J = 8.6 Hz, 2H, Ar–H), 7.15–7.18 (m, 2H, Ar–H), 7.28–7.30 (m, 3H, Ar–H), 7.73 (s, 1H, Ar–H), 8.91 (brs, 1H, NH), 9.11 (brs, 1H, NH), 10.29 (brs, 1H, NH), 10.86 (s, 1H, HCl); ^{13}C NMR (DMSO-d$_6$): δ = 21.09, 21.43, 22.38, 23.09, 30.56, 41.11, 49.01, 50.24, 51.58, 52.76, 59.33, 108.72, 112.50, 113.27, 120.03, 128.86, 139.25, 140.53, 152.87, 167.65, 171.97; HRMS [ES$^+$]: calcd for $C_{30}H_{42}Cl_2N_6O_2$, 589.5995 [M + H]$^+$, found 589.2825.

4.2.3. Synthesis of compounds 18a and 18b by Method 2

*4.2.3.1. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(dimethylamino)acetamide (**18a**).* Compound **18a** was prepared from **25c** (2.0 g, 4.5 mmol) and dimethylamine (2 M solution in THF, 2.5 mL, 5.0 mmol); Yield: 1.6 g (76%).

*4.2.3.2. N-(4-(3-(4-(bis(2-chloroethyl)amino)phenyl)ureido)phenyl)-2-(diethylamino)acetamide (**18b**).* Compound **18b** was prepared from **25c** (4.1 g, 9.25 mmol) and diethylamine (1.1 mL, 10.1 mmol); Yield: 3.7 g (83.5%).

4.3. Biological assays

4.3.1. Cytotoxicity assays

The in vitro cytotoxicity of the newly synthesized compounds against the human lymphoblastic leukemia cell line (CCRF-CEM) and its vinblastine-resistant sub-cell line (CCRF-CEM/VBL), the non-small cell lung carcinoma cell line (H1299), the prostate cancer cell line (PC3), the human lung cancer cell line (H460), and the human colon cancer cell lines (HCT-116, H3347, DLD-1, and HT-29) were determined by the Alamar Blue® (AbD Serotec, UK) assay [14] in a 72-h incubation using a microplate spectrophotometer as previously described [9,16,28]. Cell viability in HCT-116 cells treated with a combination of compound **18b** and 5-FU was assessed by seeding 3000 to 4000 HCT-116 cells in each well of a 96-well plate, followed by incubation at 37 °C in a 5% CO_2 humidified atmosphere overnight. The cells were then incubated with compound **18b** (at concentrations ranging from 0.78 μM to 100 μM), 5-FU (at concentrations ranging from 0.78 μM to 100 μM) or a combination of compound **18b** and 5-FU for 72 h. The cell viability and CI values were determined by Alamar Blue® assay and CompuSyn software as previously described [9,16,28].

4.3.2. In vivo studies

Animal care was approved by and followed the guidelines of the Academia Sinica Institutional Animal and Utilization Committee. Athymic nude mice bearing the nu/nu gene were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Male nude mice (6 weeks or older weighing 20–24 g or more) were used for all human tumor xenografts. The tested compounds were administered through tail vein iv injection as described previously [29]. Tumor volume was determined by measuring the length × width × height (or width) with calipers. The vehicle used for compounds **8**, **18a, 18b**, irinotecan, and 5-FU was a 5% dextrose isotonic solution (D5W), while oxaliplatin was prepared in saline (0.9% NaCl isotonic solution). For tumor-bearing nude mice during the course of the experiment, the % body weight changes refer to: (the total weight on reading day/the total weight on day treatment started) × 100.

4.3.3. Alkaline agarose gel shift assay

Alkaline agarose gel electrophoresis was analyzed to determine the formation of DNA cross-links as previously described [9]. Briefly, the purified pEGFP-N1 plasmid DNA (1500 ng) was mixed with the tested compounds at various concentrations (1, 2, and 4 μM) and incubated at 37 °C for 2 h. At the end of the reaction, the plasmid DNA was linearized and electrophoretically resolved on a 0.8% alkaline agarose gel with NaOH-EDTA buffer at 4 °C as previously described [9]. The DNA was visualized under UV light after staining the gels with an ethidium bromide solution.

4.3.4. Modified comet assay

Intracellular DNA interstrand cross-linking was analyzed by a modified comet assay as previously described [30]. Briefly, the growing HCT-116 cells were treated with compound **18b** for 1 h and then irradiated with 20 Gy of X-ray radiation to induce DNA strand breaks. An aliquot of 5 × 10^5 cells was subjected to single cell alkaline gel electrophoresis as previously described [30]. The level of interstrand cross-linking is proportional to the decrease in the tail moment of the irradiated drug treated sample compared to the irradiated untreated control. The decrease in the tail moment is

calculated by the following formula: % of DNA with interstrand cross-linking = [1−(TMdi−TMcu/TMci−TMcu)] × 100, where TMdi = mean tail moment of the drug-treated, irradiated sample; TMcu = mean tail moment of the unirradiated control sample; and TMci = mean tail moment of the irradiated control sample.

4.3.5. Cell cycle analysis

The effects of compounds **18b** on cell cycle progression were analyzed with a flow cytometer, as previously described [31]. Briefly, 1.5×10^5 HCT-116 cells were seeded in each well of a 6-well plate and incubated at 37 °C in a 5% CO_2 humidified atmosphere overnight. The cells were then incubated with **18b** (0.25, 0.5, 1, and 2 μM) for different periods (24, 48, and 72 h). At the end of treatment, the cell-cycle phase distribution was analyzed with FACScan flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT 3.0 software (Verity Software House, Topsham, ME) and was based on the DNA histograms.

4.3.6. Western blot analysis

HCT-116 cells were treated with compound **18b** (1, 2, and 4 μM) or oxaliplatin (50 μM). Drugs were washed out with PBS 1 h later. After a 24-h incubation, HCT-116 cells were harvested and washed twice with PBS, and the cells were then pelleted by centrifugation and lysed with Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA). Protein content was measured by absorbance at 595 nm on a microplate reader using protein dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Total protein (30 μg) was separated by SDS/PAGE and transferred to PVDF membranes (GE Healthcare, Amersham Place, UK) for protein detection. The membranes were probed with antibodies against caspase-3, c-caspase-3, PARP, c-PARP (Cell Signaling), and beta-actin (Abcam, Cambridge, MA, USA). Protein bands were visualized by the ECL detection system (Millipore Corporation, Milford, MA, USA).

4.3.7. TUNEL assay

The TUNEL assay was performed to quantify apoptotic cells in xenograft tumor sections by using a DeadEnd™ colorimetric apoptosis detection kit (Promega, G7360). Xenograft tumor sections were removed 24 h after the last treatment day, fixed with 10% neutral buffered formalin (NBF) [formaldehyde (100 mL) in H_2O (900 mL), NaH_2POH_2O (4 g), and $NaHPO_4$ (6.5 g), pH = 6.8], embedded in paraffin, sectioned, and subjected to TUNEL assay. The assay was performed according to the manufacturer's instructions. TUNEL images were acquired with a microscope linked to a digital camera (DCS0429; Kodak, Rochester, NY, USA).

4.3.8. In vivo pharmacokinetic study/drug administration and sampling of compound 18b

All animal studies were conducted in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the care and use of animals and prior approval of the Institutional Animal Care and Use Committee (IACUC), Development Center for Biotechnology (DCB), Taipei, Taiwan. A total of 6 male Sprague Dawley rats weighing 259–300 g were purchased from BioLASCO Taiwan (Taipei, Taiwan). The in-life portion of the study was conducted at the Department of Animal Pharmacology in DCB. Compounds were administered by iv bolus injection at 5.0 mg/kg ($n = 3$) or po (gavage) at 50.0 mg/kg ($n = 3$) in EtOH and normal saline (20/80, v/v). Serial blood samples (150 μL) were collected at 11 time points for po or iv into sodium heparin centrifuge tubes. Plasma concentrations were determined by LC-MS/MS (Agilent Technologies, Inc., Palo Alto, CA, USA), and data were analyzed by non-compartmental methods using WinNonlin™, version 5.3 (WinNonlin™ Copyright© 2009, Pharsight Corporation, Mountain View, CA).

4.3.9. Acute intravenous injection toxicity of compound 18b in mice

The 14-day acute toxicity of compound **18b** in mice via a single administration by iv injection was designed and conducted at the Center of Toxicology and Preclinical Sciences (CTPS), QPS, Taipei, Taiwan. Animal care was approved by and followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) Statement. Male CD-1 (ICR) mice were obtained from BioLASCO Taiwan Co. Ltd., Taipei, Taiwan. At animal randomization, the age was approximately 8–9 weeks, and the body weight range was 32.4–36.5 g. Before randomization, all animals were weighed, and clinical signs were observed. Healthy animals were selected for the study. The animals were allocated into 5 dose groups (5 males/group). The mean body weight of each group was not significantly different ($p < 0.05$) from each other following the randomization process, as determined by a one-way analysis of variance (ANOVA). The weight variation of the animals used did not exceed ±20% of the mean weight. Compound **18b** was dissolved in 20% (v/v) D-PBS and 80% (v/v) D5W to achieve the designed concentrations and given (15 mL/kg) via a single tail vein iv injection at dose levels of 55, 75, 105, and 150 mg/kg to adult ICR male mice (5/group). The control animals were given the same dose volume of vehicle control, a mixture of GIBCO® Dulbecco's PBS (Lot no.: 1233515), 20% (v/v), and 5% Dextrose (Lot no.: 1RK0401), 80% (v/v). All animals were observed for a 14-day study period after single administration of the test compound. The first dosing day was designated D1; the subsequent days were consecutively numbered. The body weights, total body weight changes, and food consumption were expressed as the mean ± standard deviation with Microsoft® Office Excel 2010. Statistical analysis was performed using SigmaStat™ Statistical Software for Windows™, Release 3.0 (Jandel Scientific Inc., USA).

4.3.10. Cardiac safety studies (hERG fluorescence polarization assay) of compound 18b

The FP assay was performed using the Predictor™ hERG fluorescence polarization assay kit (PV 5365, Invitrogen, CA, USA), which included the hERG membrane and hERG tracer red, and E-4031 was obtained from Invitrogen (Carlsbad, CA). The binding assay was conducted following the manufacturer's recommended protocol [32] with some modifications. An aliquot of 5 μL of test compound **18b** and QC control (astemizole) in FP assay buffer was added to the appropriate wells of a black 384-well micro-plate containing 10 μL of hERG membrane and 5 μL of 4 nM fluorescent tracer and covered with a plate lid to protect the reagents from exposure to light. The experiments were performed using triplicate wells in the presence or absence of excess amounts of competitor, E-4031. Following a 4 h incubation at rt, the FP was read on a Tecan Infinite® F200 plate reader (TECAN Group Ltd., Switzerland) using a polarized excitation filter setting at 535 nm and emission filter setting at 590 nm (25 and 20 nm bandwidths, respectively). Assay robustness was assessed by measuring 16 replicate positive control wells (containing 5 μL of 120 μM E-4031, 10 μL hERG membrane, and 5 μL of 4 nM tracer) and negative control wells (containing 5 μL hERG buffer, 10 μL hERG membrane, and 5 μL of 4 nM tracer). All data analyses and presentations were performed using Microsoft® Office Excel 2010 and WinNonlin™.

Acknowledgments

We are grateful for support from the National Science Council, Taiwan (Grant NSC 101-2325-B-001-037 to T.-L. Su and Grant NSC-101-2325-B-001-002 to T.-C. Lee), and Academia Sinica, Taipei,

Taiwan (Grant AS-100-TP-B13 to T.-C.L. and T.-L.S.). The NMR spectra of the synthesized compounds were obtained at the High-Field Biomolecular NMR Core Facility supported by the National Research Program for Genomic Medicine (Taiwan) and the Core Facility for Protein Structural Analysis supported by the National Core Facility Program for Biotechnology (Taiwan). The NMR spectra were acquired on a Bruker 400 MHz spectrometer at the Institute of Biological Chemistry, a Bruker 500 MHz spectrometer at the Institute of Biomedical Sciences and at the Core Facility for Protein Structural Analysis supported by National Core facility Program for Biotechnology. We are also thankful to the Development Center for Biotechnology (DCB), Taipei, Taiwan for the pharmacokinetic and hERG study. The authors also thank the Center of Toxicology and Preclinical Sciences (CTPS), QPS, Taipei, Taiwan for the acute toxicity study. The National Center for High-Performance Computing is acknowledged for our use of computer time and facilities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.018.

References

[1] N. Kapuriya, K. Kapuriya, X. Zhang, T.-C. Chou, R. Kakadiya, Y.-T. Wu, T.-H. Tsai, Y.-T. Chen, T.-C. Lee, A. Shah, Y. Naliapara, T.-L. Su, Bioorganic & Medicinal Chemistry 16 (2008) 5413–5423.
[2] N. Kapuriya, K. Kapuriya, H. Dong, X. Zhang, T.-C. Chou, Y.-T. Chen, T.-C. Lee, W.-C. Lee, T.-H. Tsai, Y. Naliapara, T.-L. Su, Bioorganic & Medicinal Chemistry 17 (2009) 1264–1275.
[3] R. Kakadiya, H. Dong, A. Kumar, N. Dodiya, N. Kapuriya, X. Zhang, T.-C. Chou, T.-C. Lee, A. Shah, T.-L. Su, Bioorganic & Medicinal Chemistry 18 (2010) 2285–2299.
[4] W.D. Kingsbury, J.C. Boehm, D.R. Jakas, K.G. Holden, S.M. Hecht, G. Gallagher, M.J. Caranfa, F.L. McCabe, L.F. Faucette, R.K. Johnson, R.P. Hertzberg, Journal of Medicinal Chemistry 34 (1991) 98–107.
[5] S. Sawada, S. Okajima, R. Aiyama, K. Nokata, T. Furuta, T. Yokokura, E. Sugino, K. Yamaguchi, T. Miyasaka, Chemical & Pharmaceutical Bulletin 39 (1991) 1446–1454.
[6] R. Kath, K. Blumenstengel, H.J. Fricke, K.J. Höffken, Journal of Cancer Research and Clinical Oncology 127 (2001) 48–54.
[7] A.V. Patterson, D.M. Ferry, S.J. Edmunds, Y. Gu, R.S. Singleton, K. Patel, S.M. Pullen, K.O. Hicks, S.P. Syddall, G.J. Atwell, S. Yang, W.A. Denny, W.R. Wilson, Clinical Cancer Research 13 (2007) 3922–3932.
[8] G. Nagaiah, S.C. Remick, Future Oncology 6 (2010) 1219–1228.
[9] N. Kapuriya, R. Kakadiya, H. Dong, A. Kumar, P.-C. Lee, X. Zhang, T.-C. Chou, T.-C. Lee, C.-H. Chen, K. Lam, B. Marvania, A. Shah, T.-L. Su, Bioorganic & Medicinal Chemistry 19 (2011) 471–485.
[10] E.C. Bigham, S.J. Hodson, W.R. Mallory, D. Wilson, D.S. Duch, G.K. Smith, R. Ferone, Journal of Medicinal Chemistry 35 (1992) 1399–1410.
[11] K.E. Andersen, B.F. Lundt, A.S. Jorgensen, C. Braestrup, European Journal of Medicinal Chemistry 31 (1996) 417–425.
[12] I. Sestili, A. Borioni, C. Mustazza, A. Rodomonte, L. Turchetto, M. Sbraccia, D. Riitano, M.R. Del Giudice, European Journal of Medicinal Chemistry 39 (2004) 1047–1057.
[13] W.C. Drewe, R. Nanjunda, M. Gunaratnam, M. Beltran, G.N. Parkinson, A.P. Reszka, W.D. Wilson, S. Neidle, Journal of Medicinal Chemistry 51 (2008) 7751–7767.
[14] S. Al-Nasiry, M. Hanssens, C. Luyten, R. Pijnenborn, Human Reproduction 22 (2007) 1304–1309.
[15] A. de Gramont, A. Figer, M. Seymour, M. Homerin, A. Hmissi, J. Cassidy, C. Boni, H. Cortes-Funes, A. Cervantes, G. Freyer, D. Papamichael, N. Le Bail, C. Louvet, D. Hendler, F. de Braud, C. Wilson, F. Morvan, A. Bonetti, Journal of Clinical Oncology 18 (2000) 2938–2947.
[16] T.-C. Chou, Pharmacological Reviews 58 (2006) 621–681.
[17] T.-C. Chou, Cancer Research 70 (2010) 440–446.
[18] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, Experimental Cell Research 175 (1988) 184–191.
[19] B.J. Friedmann, M. Caplin, B. Savic, T. Shah, C.J. Lord, A. Ashworth, J.A. Hartley, D. Hochhauser, Molecular Cancer Therapeutics 5 (2006) 209–218.
[20] V. Moinet-Hedin, T. Tabka, L. Poulain, T. Godard, M. Lechevrel, C. Saturnino, J.C. Lancelot, J.Y. Le Talaer, P. Gauduchon, Anti-Cancer Drug Design 15 (2000) 109–118.
[21] A.J. Polverino, S.D. Patterson, The Journal of Biological Chemistry 272 (1997) 7013–7021.
[22] P.J. Duriez, G.M. Shah, Biochemistry and Cell Biology 75 (1997) 337–349.
[23] S.H. Kaufmann, S. Desnoyers, Y. Ottaviano, N.E. Davidson, G.G. Poirier, Cancer Research 53 (1993) 3976–3985.
[24] D.R. Piper, S.R. Duff, H.C. Eliason, W.J. Frazee, E.A. Frey, M. Fuerstenau-Sharp, C. Jachec, B.D. Marks, B.A. Pollok, M.S. Shekhani, D.V. Thompson, P. Whitney, K.W. Vogel, S.D. Hess, ASSAY and Drug Development Technologies 6 (2008) 213–223.
[25] M.C. Sanguinetti, M. Tristani-Firouzi, Nature 440 (2006) 463–469.
[26] P.L. Hedley, P. Jorgensen, S. Schlamowitz, R. Wangari, J. Moolman-Smook, P.A. Brink, J.K. Kanters, V.A. Corfield, M. Christiansen, Human Mutation 30 (2009) 1486–1511.
[27] L.N. Owen, J.F. Danielli, Nitrogen Mustards, GB915454, 1960; [Chem. Abstr. 58 (1963) 12464].
[28] T.-C. Chou, N. Martin, CompuSyn Software, ComboSyn, Inc., Paramus, NJ, 2005.
[29] T.-C. Chou, O.A. O'Connor, W.P. Tong, Y. Guan, Z.-G. Zhang, S.J. Stachel, C. Lee, S.J. Danishefsky, Proceedings of the National Academy of Sciences 98 (2001) 8113–8118.
[30] R. Kakadiya, H. Dong, P.-C. Lee, N. Kapuriya, X. Zhang, T.-C. Chou, T.-C. Lee, K. Kapuriya, A. Shah, T.-L. Su, Bioorganic & Medicinal Chemistry 17 (2009) 5614–5626.
[31] T.C. Lee, J.L. Ko, K.Y. Jan, Toxicology 56 (1989) 289–299.
[32] Predictor hERG FP assay Manual, http://tools.invitrogen.com/content/sfs/manuals/Predictor_hERG_FP_Assay_man.pdf.