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Synthesis and Antitumor Activity of Novel Nitrogen Mustard-Linked Chalcones

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A series of nitrogen mustard-linked chalcones were synthesized and evaluated for their antitumor activity *in vitro* against the K562 and HepG2 cell lines. The aldol condensation of [*N*,*N*-bis(chloroethyl)-3-amino]-acetophenone (**2**) with aromatic aldehydes afforded the nitrogen mustard-linked chalcones. Among the analogs tested, compounds **5e** and **5k** exhibited significant anti-proliferation activities against K562 cells with IC_{50} values of 2.55 and 0.61 μM, respectively, which revealed higher cell toxicity than the standard drugs cisplatin ($IC_{50} > 200$ μM) and adriamycin ($IC_{50} = 14.88$ μM). The methoxyl and *N*,*N*-dimethyl groups on the B-ring of the chalcone frame enhanced the inhibitory activities against both the K562 and HepG2 cell lines. The structure–activity relationship study indicated that the inhibitory activities significantly varied with the position(s) and species of the substituted group(s).

Keywords: Anti-proliferation / Cell toxicity / Chalcones / Crystal structure / Nitrogen mustard

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Introduction

Chalcones are a group of natural compounds belonging to the flavonoid family and are found in the heartwood, bark, leaves, fruits, and roots of a variety of trees and plants [1]. Chemically, with a common 1,3-diphenyl-2-propen-1-one framework (Fig. 1), chalcones, as the main precursors in the biosynthesis of flavonoids and as an important substructure of some natural compounds, have been known for over a century. Up to date, many studies have shown that the natural and synthetic chalcone derivatives show a diverse array of biological activities, among which are antioxidant [2], antimalarial [3], anti-inflammatory [4], antileishmanial [5], antibacterial, and antifungal activities [6, 7]. Recent reports indicated that chalcones, as a kind of anticancer agents [8, 9], exhibit potential cytotoxicity in the treatment of some kinds of cancer neoplastic diseases. Wang et al. [10] have shown that millepachine **1** (Fig. 2), which was isolated from *Millettia pachycarpa* for the first time in their group, exhibited potent apoptosis-inducing effects, making them promising candidates for the treatment of cancer. Nakamura et al. [11] have designed and synthesized fluorinated 3,4-dihydroxychalcone **2**, which showed 5-lipoxygenase inhibition on rat basophilic leukemia-1 (RBL-1) cells and inhibitory action on Fe^{3+}-ADP-induced NADPH-dependent lipid peroxidation in rat liver microsomes. Xia et al. [12] synthesized the novel 2′-amino chalcone **3**, which had high activity towards the multi-drug resistant KB-VIN and the ovarian 1A9 cell lines, with IC_{50} values of 0.30 and 0.35 μg/mL, respectively. In order to investigate and develop efficient and broad-spectrum antitumor drugs, the structure modification of chalcones has become a focus in related research in the recent years.

Bifunctional alkylating agents, particularly nitrogen mustards, are an important class of anticancer drugs [13, 14]. With the

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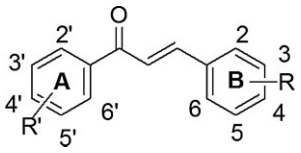


Figure 1. The basic framework of chalcones.

N,*N*-bis(2-chloroethyl)amine pharmacophore, nitrogen mustards are believed to exert their biological activity through interstrand cross-linking in the major groove of DNA (Scheme 1) [13, 15], and this linkage represents the highest toxicity of all alkylation events. However, due to their high reactivity and the fact that they have no particular affinity for alkylating DNA, a high proportion of those nitrogen mustards drugs can easily react with other cellular components such as proteins [16], thiols, or genes [17] before reaching the DNA. And this disadvantage produced many unwanted side effects such as bone marrow toxicity [18], leading to the loss of the drugs' therapeutic activity against malignancy. Also, a significant amount of the drug may reach the DNA with only one arm of the mustard intact, forming the monoadducts that are considered to be more genotoxic rather than toxic [19] (Scheme 1). Drugs of this class, such as mechlorethamine [20], melphalan [21], and chlorambucil [22], are widely used in cancer treatment, and their cytotoxicity appears to be related to the extent of their interstrand cross-linking with DNA. Like other bifunctional alkylating agents, they are active against both resting and rapidly dividing tumor cells [23].

In the past several years, designs and modifications of the structure of nitrogen mustards to increase their cytotoxicity, especially for their specificity toward tumor cells, have been in the focus of interest of many biochemists [24–26]. But, to the best of our knowledge, there have so far been no literature reports regarding nitrogen mustard-containing chalcone derivatives in which the *N*,*N*-bis(2-chloroethyl)-amine pharmacophore was linked to the chalcone framework. In our present study, we designed and synthesized a series of nitrogen mustard-linked chalcone derivatives, and their anticancer activity against two cancer cell lines was evaluated *in vitro*, with the aim of searching new potent and selective anticancer agents. We also elucidated the influence of the electronic effect of different substituents on the chalcone framework on the cytotoxicity of the chalcones.

Results and discussion

Synthesis

The newly synthesized compounds are nitrogen mustards linked to substituted chalcones. In order to investigate the structure–activity relationship (SAR) of the chalcone-nitrogen mustard conjugates, we introduced several different

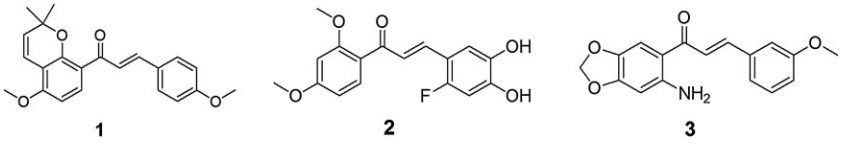
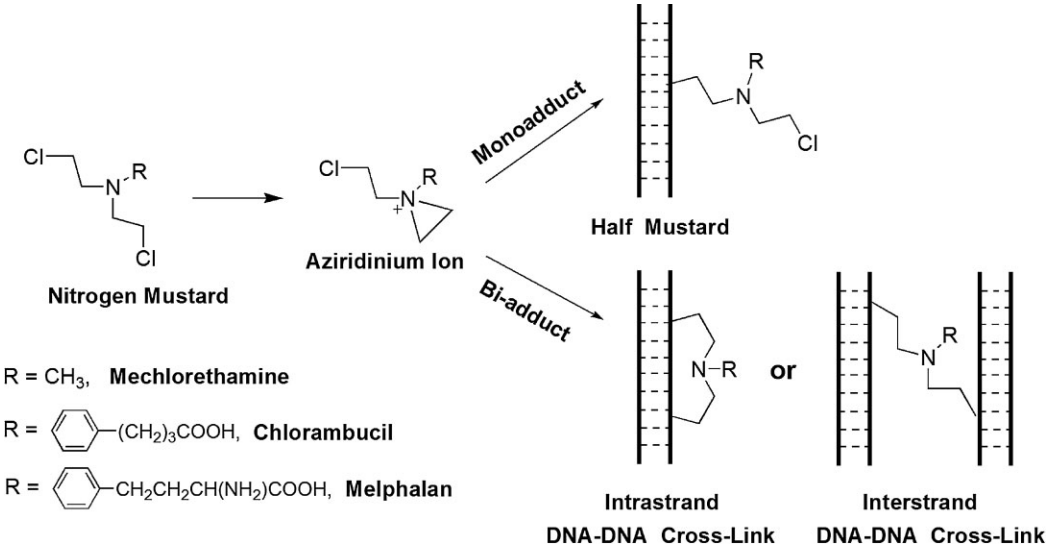


Figure 2. Structures of some reported chalcones with antitumor potency.



Scheme 1. Chemical structures of nitrogen mustards and formation of DNA-mustard cross-links.

substituents into the B-ring of the chalcone framework. The novel nitrogen mustard-linked chalcone derivatives **5a–5k** were obtained through the synthetic route shown in Scheme 2. First, 3-nitroacetophenone was reduced by $Fe/NH_4Cl–CH_3COOH$ in ethanol, giving 3-aminoacetophenone **1** [27]. Hydroxyethylation of **1** with 2-chloroethanol in water and in the presence of $CaCO_3$ at 120°C for 7 h afforded 3-[*N*,*N*-bis(2-hydroxyethyl)-amino]-acetophenone **2** [28]. In order to obtain the intermediate 3-[*N*,*N*-bis(2-hydroxyethyl)-amino]-acetophenone **3**, we first attempted the method of direct chlorination by treating the diol **2** with $SOCl_2$ in CH_2Cl_2 or by treating with $POCl_3$ [29, 30]. Unfortunately, this did not give the required chlorinated compound. So we adopted the indirect chlorination method: Tosylation of diol **2** followed by displacement with NaCl in DMF [31] gave the intermediate **4**. Compounds **5a–k** were obtained by a base-catalyzed Claisen–Schmidt condensation [32] of **3** with substituted aromatic aldehydes. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures. The IR and ^1H NMR spectra of compounds **1–4** and **5a–k** are given in Supporting Information Figures S1–S30.

Crystallographic structures of compounds 3 and 5c

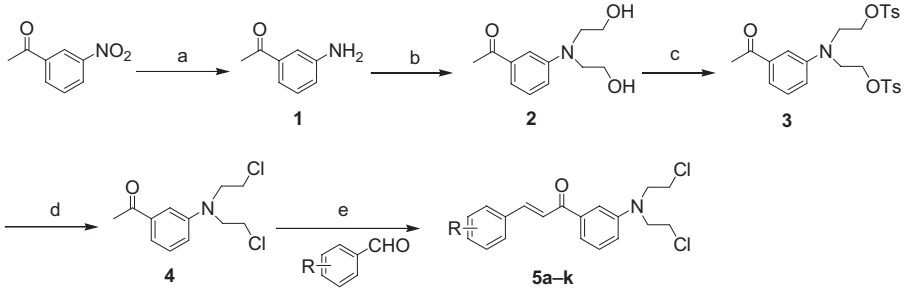
Suitable crystals of compounds **3** and **5c** for X-ray diffraction (XRD) were grown by the solvent evaporation method. The diffraction intensity data were collected on a Bruker Smart Apex II CCD diffractometer with graphite-monochromated Mo Kα radiation ($k = 0.71073$) at 296(2) K. Empirical absorption corrections were applied using the SADABS program. The structures were solved by direct methods and refined by the full-matrix least squares based on F^2 using the SHELXTL-97 program. All non-hydrogen atoms were refined anisotropically and hydrogen atoms were generated geometrically. The crystal data and structural refinement parameters of **3** and **5a** are summarized in Supporting Information Table S1. The two molecular structures with the atom numbering scheme are shown in Fig. 3.

Compounds **3** and **5c** crystallized in different space groups, $P2_1/n$ and *P*-1. XRD studies on compound **3** showed that the two benzene rings derived from the *p*-toluenesulfonyl unit are planar, exist on both sides of the benzene ring plane [C11, C12, C13, C14, C15, C16, C17] and are almost parallel with it (Fig. 2). The dihedral angles between the three benzene rings are 5.27°, 11.22°, and 5.91°, respectively. There are similarities in the bond lengths of S(1)–O(3) and S(2)–O(5), with distances of 1.573(2) and 1.570(2) Å, respectively, because of the existence of the same electronegativity and space volume. In the crystal lattice, there are several weak hydrogen bond intramolecular interactions (Supporting Information Table S2) and the crystal packing is further stabilized by these hydrogen bonds.

XRD studies were also carried out for the title compound **5c**. The two benzene rings are almost co-planar, with dihedral angles of 4.9(42)°. The bond lengths and angles of the benzene rings are quite unequivalent, which may be the result of the different space volumes and electronegativities of the substituents in each ring. There are several weak intramolecular hydrogen bonds (Supporting Information Table S3).

Biological activity tests

The cytotoxicities of the compounds were evaluated against the human cancer cell lines HepG2 (liver cells) and K562



5a: R= 2-N(CH_2CH_2Cl)$_2$ **5b:** R= 2,4-Cl_2 **5c:** R= p-NO_2 **5d:** R= p-Br
5e: R= p-OCH_3 **5f:** R= 2-F **5g:** R= H **5h:** R= o-Cl
5i: R= p-F **5j:** R= p-Cl **5k:** R= p-N(CH_3)$_2$

Reagents and conditions: (a) Fe powder, NH_4Cl, CH_3COOH, ethanol, reflux, 2 h; (b) 2-chloroethanol, $CaCO_3$, H_2O, 120°C, 7 h; (c) TsCl, Et_3N, THF, CH_2Cl_2, r.t., 26 h; (d) DMF, LiCl, 110°C, 30 min; (e) substituted aromatic aldehyde, ethanol, 2.5 M NaOH, r.t., 4–6 h.

Scheme 2. Synthesis of nitrogen mustard-linked chalcone derivatives **5a–5k**.

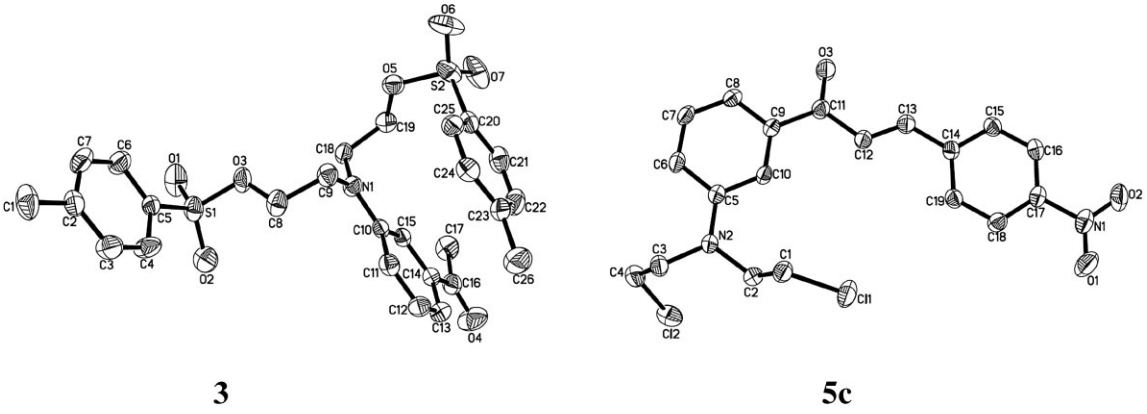


Figure 3. ORTEP diagrams for compounds **3** and **5c**.

(human leukemia cells) by the colorimetric MTT assay [33, 34]. Although their binding sites might not be identical with those of the series of nitrogen mustard-linked chalcone derivatives, the anticancer drugs cisplatin (DDP) and adriamycin (ADM) were used as reference compounds because of their well-established clinical application in cancer. The results are presented in Table 1, which illustrates the concentrations required to inhibit cell growth by 50% (IC_{50} values); a low IC_{50} is desired and implies cytotoxicity or anti-proliferation activity at low drug concentrations [35, 36].

The anti-proliferation activity results for the K562 cells treated with each compound indicate that compound **5k** (4-N$(CH_3)_2$) showed the highest activity with an IC_{50} value of 0.61 μM. The compounds displaying a potent anti-proliferation effect on K562 cells were **5a** (4-N$(CH_2CH_2Cl)_2$), **5b** (2,4-di-Cl), **5c** (4-NO_2), **5d** (4-Br), **5e** (4-OCH_3), **5f** (2-F), and **5g**, with IC_{50} values of 6.53, 10.39, 10.82, 13.43, 2.55, 4.22, and 14.75 μM, respectively. They all revealed higher activity than the standard drug adriamycin (IC_{50} = 14.88 μM). Compounds **5h** (2-Cl), **5i** (4-F), and **5j** (4-Cl) were as active as the control drug (adriamycin) or less active than it. All the newly synthesized chalcones displayed much higher potency than the other standard drug, cisplatin (IC_{50} > 200 μM). As we can see, replacement of the 4-H of the B-ring with substituents such as *N*,*N*-dimethyl (**5k**), nitro (**5c**), a bromine atom (**5d**), methoxyl (**5e**), and the *N*,*N*-dichloroethyl group (**5a**) displayed different degrees of increase in their anti-proliferation activity against K562 cells. In contrast, groups as fluorine and chlorine atoms introduced at the *para*-position of the B-ring may decrease the cytotoxicity on the selected cell lines. Turning to the effects of an electron-releasing group at the *para*-position, we found that replacement of electron-withdrawing groups with electron-donating groups (**5a**, **5k**) causes an increase in cytotoxicity on the K562 cell line. Moreover, comparison of the cytotoxicities of **5b**, **5h** and **5j**, **5f** and **5i**, respectively, led to the impression that a fluorine or chlorine atom at the *ortho*-position of the B-ring, instead of the same atom at the *para*-position, may enhance the cytotoxicity on the K562 cell line.

Table 1. IC_{50}[a] (μM) values of the target compounds for K562 and HepG2 cells.

Compound	K562	HepG2
5a	6.53	34.98
5b	10.39	44.24
5c	10.82	24.97
5d	13.43	41.66
5e	2.55	5.19
5f	4.22	15.22
5g	14.75	26.50
5h	15.06	18.97
5i	16.73	16.06
5j	16.57	15.60
5k	0.61	8.53
ADM	14.88	4.19
DDP	N[b]	11.31

[a] IC_{50} values are the mean of three experiments in replicate of at least three independent experiments.
[b] N means inactive (IC_{50} values > 200 μM).

In HepG2 cells, the results followed a different trend: Compound **5e** was a good substrate for inhibition of these cells, providing the lowest IC_{50} value (5.19 μM) among the newly prepared chalcones. However, replacement of 4-H of **5g** with the electron-withdrawing groups mentioned before resulted in an increase in cytotoxicity on HepG2 cells to a minor degree. When comparing the cytotoxicities of compounds with groups as the fluorine or chlorine atom at the *ortho*- and *para*-positions of the B-ring, we found that only fluorine in the *ortho*-position caused a minor increase in anti-proliferation activity, while the chlorine atom had the opposite effect. Compounds **5i**, **5j**, **5f**, and **5k** showed moderate to good cytotoxicity on the HepG2 cell line, with IC_{50} values of 16.06, 15,60, 15.22, and 8.54 μM, respectively, and the

cytotoxicities on HepG2 cells of these compounds were close to that of the standard drug cisplatin (IC_{50} = 11.31 μM), but higher than that of adriamycin (IC_{50} = 4.19 μM). More impressively, the chalcones **5e** and **5k** with the methoxyl and *N*,*N*-dimethyl group at the *para*-position of the B-ring, respectively, showed high potency in cytotoxicity on both the K562 and HepG2 cell lines, suggesting that these kinds of compounds are of value in the future as drugs for chemotherapy.

These results are consistent with previous reports on SAR studies on chalcones derivatives, which showed that even a subtle modification of the chalcone chemical structure may result in significant changes in their biological potency [37]. Taking into account the cytotoxicity data of this study on both the K562 and HepG2 cell lines, all the tested chalcones present moderate to good toxicity, which may indicate that this kind of compounds is worthy of further investigation.

Conclusion

In this paper, we reported on the synthesis, characterization, and antitumor activities of nitrogen mustard-linked chalcones analogs. The crystal structures of the intermediate **3** and the title compound **5a** were obtained, and structural analysis revealed that compounds **3** and **5a** are monoclinic with the space group $P2_1/c$ and triclinic with the space group *P*-1, respectively. The SAR results revealed that the inhibition activities of this kind of compounds are dependent on the substituents on the B-ring; the structure modification indicated that a methoxyl or an *N*,*N*-dimethyl group at the *para*-position of the B-ring may increase the inhibitory activity. According to the MTT assay, most of these derivatives had micromolar-level potency against the tested cell lines. Compounds **5e** and **5k** showed the most potent inhibition, with IC_{50} values of 2.55 and 5.19 μM for K562 cells and 0.61 and 8.53 μM for HepG2 cells, respectively, revealing much higher inhibitory potency than the standard drug cisplatin and similar inhibition capacity with regard to adriamycin. The results of this study show that this kind of compounds may be considered as potential anticancer agents, and further studies need to be conducted to reach a better understanding of the signaling mechanisms of the subject compounds, and the relevant study is underway.

Experimental

Chemistry

Melting points are uncorrected and were taken on a XT-4 micro melting point apparatus. ^{1}H NMR spectra were recorded on an INOVA-400 NMR spectrometer and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane as internal standard. The infrared spectra were recorded on an EQUINOX-55 FTIR spectrometer using KBr pellets and values are expressed in cm^{-1}. ESI-MS data were recorded on an AXIMA-CFR^{TM} plus MALDI-TOF mass spectrometer. Column flash chromatography was carried out on Merck silica gel (250–400 mesh ASTM). Thin-layer chromatography (TLC) was performed on silica gel GF254. The solvents and chemicals used for synthesis were commercially available and were used without further purification unless otherwise noted.

*3-Aminoacetophenone (**1**)*

Iron powder (3.9 g, 69 mmol) was activated by refluxing it with a mixture of 10 mL distilled water, ethanol (40 mL), glacial acetic acid (1 mL), and NH_4Cl (0.2 g, 4 mmol) for 0.5 h under vigorous stirring at 80°C; then, ethanol (10 mL) and 3-nitroacetophenone (1.65 g, 10 mmol) were added to the mixture within 10 min. After the mixture was heated slowly until reflux under vigorous stirring for 2 h, the hot reaction mixture was filtered and the residue was washed with ethanol for three times. After evaporation of the alcohol, the residue was diluted with water and extracted with $CHCl_3/CH_3OH$ 2:1 for three times; the extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated at reduced pressure. The crude was purified on a flash column (CH_3CO_2Et/petroleum ether 2:1 as eluent) to give 3-aminoacetophenone **1**. Yield: 84% of white solid, mp 96–97°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (dd, 1H, J = 7.6, 0.8 Hz, ArH), 7.24 (t, 2H, J = 7.8 Hz, ArH), 6.89–6.84 (m, 1H, ArH), 3.90 (s, 2H, $-NH_2$), 2.55 (s, 3H, $-CH_3$); IR (KBr) ν (cm^{-1}): 3467, 3369, 3222, 1668, 1628, 1597, 1490, 1457, 1356, 1324, 1236, 777, 683.

*[N,N-Bis(2-hydroethyl)-3-amino]-acetophenone (**2**)*

A mixture of 3-aminoacetophenone (6.4 g, 47.5 mmol), 2-chloroethanol (16 mL, 240 mmol), and $CaCO_3$ (6.5 g, 65 mmol) in water (60 mL) was heated under reflux with vigorous stirring for 7 h. After hot filtering, the unreacted $CaCO_3$ was washed with a few portions of hot water; then, the filtrate was extracted with CH_2Cl_2 (40 mL × 3) dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to afford a yellow oil. The residue was further purified by SiO_2 flash column chromatography (CH_3CO_2Et/petroleum ether 2:1 as eluent) to give the required diol **2**. Yield: 68% of faint yellow liquid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.20–7.24 (m, 3H, ArH), 6.85–6.87 (m, 1H, ArH), 4.74 (s, 2H, –OH), 3.77 (s, 4H, $-OCH_2-$), 3.53–3.54 (d, 4H, J = 4.8 Hz, $-NCH_2-$), 2.49 (s, 3H, $-CH_3$); IR (KBr) ν (cm^{-1}): 3378, 2933, 2880, 1673, 1597, 1494, 1444, 1357, 1267, 1178, 1010, 779, 688; HRMS (ESI) $C_{12}H_{17}NO_3$ $[M+Na]^+$: 246.1101, found: 246.1105.

*N,N-Bis[2-(tosyloxyethyl)-3-amino]-acetophenone (**3**)*

A solution of diol **2** (5.8 g, 26 mmol) in dry THF/CH_2Cl_2 (208 mL, THF/CH_2Cl_2 1:3) was cooled at 0°C, and Et_3N (10.27 mL, 77.35 mmol) was added, followed by tosylchloride (16.65 g, 87 mmol). The mixture was stirred at r.t. for 26 h, diluted with CH_2Cl_2 and washed many times with aqueous $NaHCO_3$ and brine. After drying with anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure. The residue was chromatographed on silica gel (CH_3CO_2Et/petroleum ether 1:5 as eluent) to give the required ditosylate. Yield: 46% of faint yellow solid, mp 87–89°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (d, 4H, J = 8.2 Hz, ArH), 7.28–7.22 (m, 6H, ArH), 7.03 (d, 1H, J = 1.0 Hz, ArH), 6.68 (dd, 1H, J = 7.9, 2.0 Hz, ArH), 4.11 (t, 4H, J = 5.7 Hz, $-OCH_2-$), 3.60 (t, 4H, J = 5.7 Hz, $-NCH_2-$), 2.55 (s, 3H, $-CH_3$), 2.39 (s, 6H, $-PhC\underline{H}_3$); IR (KBr) ν (cm^{-1}): 3426, 2959, 2923, 1678, 1589, 1515, 1356, 1173, 1178, 1092, 812, 764; HRMS (ESI) calcd. for $C_{26}H_{29}NO_7S2$ $[M+Na]^+$: 554.1278, found: 554.1270.

*[N,N-Bis(2-chloroxyethyl)-3-amino]-acetophenone (**4**)*

A solution of ditosylate **3** (2.5 g, 4.7 mmol) in a minimum volume of dry DMF was added to a LiCl solution (0.8 g, 18.8 mmol) also in DMF, and the resulting mixture was heated at 110°C for 30 min. The mixture was diluted with water and extracted with CH_2Cl_2 for three times. The organic phase was washed with water and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to give a yellow residue, which was then chromatographed on silica gel (CH_3CO_2Et/petroleum ether 1:15 as eluent) to give compound **4**. Yield: 68% of light green solid, mp 55–57°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37–7.30 (m, 2H, ArH), 7.27 (s, 1H, ArH), 6.93–6.87 (m, 1H, ArH), 3.78 (t, 4H, J = 6.8 Hz, $ClCH_2$–), 3.65 (t, 4H, J = 6.8 Hz, –NCH_2–), 2.58 (s, 3H, –CH_3). IR (KBr) ν (cm^{-1}): 3084, 2962, 2923, 1670, 1593, 1557, 1519, 1399, 1358, 1164, 814, 747; HRMS (ESI) calcd. for $C_{12}H_{15}Cl_2NO$ $[M+Na]^+$: 282.0423, found: 282.0449.

*General procedure for the preparation of compounds **5a–k***

To a solution of [*N*,*N*-bis(2-chloroxyethyl)-3-amino]-acetophenone (3 mmol) and substituted aromatic aldehydes (3 mmol) in ethanol (9 mL), a solution of 2.5 M sodium hydroxide (1 mL) was added slowly within 10 min, in an ice bath. After stirring for 4–6 h at room temperature, the formed precipitate was left. The mixture was extracted with CH_2Cl_2 (10 mL × 3) and dried over $MgSO_4$. After the solvent was evaporated under reduced pressure, the crude product was obtained; then, the crude product was further purified by SiO_2 flash column chromatography. The yield, melting point, and spectral date of each compound were collected as described below.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-{4-[N,N-bis(2-chloroxyethyl)phenyl]}prop-2-en-1-one (**5a**)*

Yield: 84% of deep yellow solid, mp 124–126°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, 1H, J = 15.5 Hz, H17), 7.56 (d, 2H, J = 8.5 Hz, ArH), 7.42–7.27 (m, 4H, ArH and H17), 6.90 (d, 1H, J = 3.2 Hz, ArH), 6.70 (d, 2H, J = 8.6 Hz, ArH), 3.81 (d, 8H, J = 4.0 Hz, $ClCH_{20}$–), 3.67 (d, 8H, J = 3.3 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2923, 2855, 1648, 1570, 1519, 1443, 1351, 1164, 816, 769; HRMS (ESI) calcd. for $C_{23}H_{26}Cl_4N_2O$ $[M+Na]^+$: 511.0664, found: 511.0679.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(2,4-dichloro-phenyl)prop-2-en-1-one (**5b**)*

Yield: 87% of yellow powdery solid, mp 109–110°C; ^{1}H NMR (400 MHz, $CDCl_3$): 8.09 (d, 1H, J = 15.7 Hz, H17), 7.68 (d, J = 8.4 Hz, 1H, ArH), 7.54–7.27 (m, 6H, H16 and ArH), 6.94 (d, 1H, J = 6.2 Hz, ArH), 3.89–3.77 (m, 4H, $ClCH_2$–), 3.73–3.61 (m, 4H, –NCH_2–); IR (KBr) ν (cm^{-1}): 2922, 1656, 1582, 1457, 1357, 1312, 1250, 821, 774, 698; HRMS (ESI) calcd. for $C_{21}H_{17}Cl_4NO$ $[M+Na]^+$: 463.9929, found: 463.9933.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-nitrophenyl)prop-2-en-1-one (**5c**)*

Yield: 89% of orange red solid, mp 140–142°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, 2H, J = 8.4 Hz, ArH), 7.80 (dd, 3H, J = 11.7, 7.5 Hz, ArH), 7.61 (d, 1H, J = 15.7 Hz, H17), 7.38 (t, 3H, J = 15.6 Hz, H17 and ArH), 6.97 (d, 1H, J = 6.2 Hz, ArH), 3.89–3.79 (m, 4H, $ClCH_2$–), 3.69 (t, 4H, J = 6.3 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2923, 2855, 1664, 1600, 1563, 1507, 1452, 1340, 1249, 833, 789, 744; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_2N_2O_3$ $[M+Na]^+$: 415.0587, found: 415.0611.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-bromophenyl)prop-2-en-1-one (**5d**)*

Yield: 78% of yellow solid, mp 76–78°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (d, 1H, J = 15.7 Hz, H17), 7.51 (dt, 4H, J = 15.8, 9.0 Hz, H16 and ArH), 7.42–7.35 (m, 2H, ArH), 7.35–7.27 (m, 2H, ArH), 6.95–6.89 (m, 1H, ArH), 3.83–3.77 (m, 4H, $ClCH_2$–), 3.66 (dd, 4H, J = 15.7, 6.9 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2959, 1674, 1595, 1562, 1489, 1440, 1452, 1351, 1277, 833, 777, 727; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_2BrNO$ $[M+Na]^+$: 447.9841, found: 447.9846.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-methoxyphenyl)prop-2-en-1-one (**5e**)*

Yield: 82% of yellow solid, mp 123–124°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 1H, J = 15.6 Hz, H17), 7.59 (d, 2H, J = 8.5 Hz, ArH), 7.36 (dd, 4H, J = 18.8, 14.2 Hz, H16 and ArH), 6.93 (d, 3H, J = 8.6 Hz, ArH), 3.84 (s, 3H, –OCH_3), 3.82–3.77 (m, 4H, $ClCH_2$–), 3.66 (t, 4H, J = 6.3 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2924, 2850, 1651, 1594, 1563, 1506, 1428, 1260, 826, 785, 724; HRMS (ESI) calcd. for $C_{20}H_{21}Cl_2NO_2$ $[M+Na]^+$: 400.0842, found: 400.0862.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(2-fluorophenyl)prop-2-en-1-one (**5f**)*

Yield: 85% of yellow solid, mp 85–86°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, 1H, J = 15.9 Hz, H17), 7.63 (dd, 2H, J = 19.3, 11.6 Hz, H16 and ArH), 7.44–7.29 (m, 4H, ArH), 7.23–7.09 (m, 2H, ArH), 6.93 (d, 1H, J = 2.8 Hz, ArH), 3.81 (t, 4H, J = 6.6 Hz, $ClCH_2$–), 3.68 (t, 4H, J = 6.5 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2922, 2578, 1654, 1599, 1564, 1491, 1450, 1352, 1219, 840, 745, 695; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_2FNO$ $[M+Na]^+$: 388.0642, found: 388.0665.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-phenylprop-2-en-1-one (**5g**)*

Yield: 85% of yellow sticky liquid; ^{1}H NMR (400 MHz, $CDCl_3$): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, 1H, J = 15.9 Hz, H17), 7.63 (dd, 2H, J = 19.3, 11.6 Hz, H16 and ArH), 7.44–7.29 (m, 4H, ArH), 7.23–7.09 (m, 2H, ArH), 6.93 (d, J = 2.8 Hz, 1H, ArH), 3.81 (t, 4H, J = 6.6 Hz, $ClCH_2$–), 3.68 (t, 4H, J = 6.5 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 3028, 2958, 2924, 1658, 1596, 1491, 1446, 1351, 1292, 1214, 756, 687; HRMS (ESI) calcd. for $C_{19}H_{19}Cl_2NO$ $[M+Na]^+$: 370.0736, found: 370.0749.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(2-chlorophenyl)prop-2-en-1-one (**5h**)*

Yield: 86% of yellow solid, mp 85–86°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (d, 1H, J = 15.8 Hz, H17), 7.73 (s, 1H, ArH), 7.44 (d, 2H, J = 15.7 Hz, H16 and ArH), 7.34 (d, 5H, J = 21.2 Hz, ArH), 6.93 (s, 1H, ArH), 3.81 (t, 4H, J = 6.4 Hz, $ClCH_2$–), 3.68 (d, 4H, J = 6.5 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 3068, 2959, 2924, 1662, 1593, 1489, 1443, 1353, 1275, 1212, 1045, 756, 691; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_3NO$ $[M+Na]^+$: 404.0346, found: 404.0359.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-fluorophenyl)prop-2-en-1-one (**5i**)*

Yield: 86% of pale yellow solid, mp 65–67°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (d, 1H, J = 15.7 Hz, H16), 7.57 (d, 2H, J = 8.4 Hz, ArH), 7.47 (d, 2H, J = 15.7 Hz, H17 and ArH), 7.39 (dd, 3H, J = 7.3, 4.1 Hz, ArH), 7.32 (s, 1H, ArH), 6.94 (dd, 1H, J = 5.9, 3.2 Hz, ArH), 3.82 (t, 4H, J = 6.8 Hz, $ClCH_2$–), 3.68 (t, 4H, J = 6.8 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2924, 2857, 1658, 1589, 1502, 1448, 1446, 1211, 825, 780, 735; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_2FNO$ $[M+Na]^+$: 388.0642, found: 388.0665.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-chlorophenyl)prop-2-en-1-one (**5j**)*

Yield: 92% of pale yellow solid, mp 103–105°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, 1H, J = 15.7 Hz, H17), 7.69–7.60 (m, 2H, H16 and ArH), 7.49–7.30 (m, 4H, ArH), 7.11 (t, 2H, J = 8.5 Hz, ArH), 6.93 (dd, 1H, J = 5.7, 3.1 Hz, ArH), 3.82 (t, 4H, J = 6.7 Hz, $ClCH_2$–), 3.68 (t, 4H, J = 6.7 Hz, –NCH_2–); IR (KBr) ν (cm^{-1}): 2923, 2855, 1654, 1596, 1560, 1486, 1391, 1276, 820, 769, 722; HRMS (ESI) calcd. for $C_{19}H_{18}Cl_3NO$ $[M+Na]^+$: 404.0346, found: 403.0364.

*(E)-3′-[N,N-Bis(2-chloroxyethyl)-amino-phenyl]-1-(4-dimethylaminophenyl)prop-2-en-1-one (**5k**)*

Yield: 87% of orange-yellow solid, mp 105–107°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, 1H, J = 15.4 Hz, H17), 7.54 (d, 2H, J = 8.2 Hz, ArH), 7.34 (d, 4H, J = 23.5 Hz, ArH and H16), 6.89 (s, 1H, ArH), 6.68 (d, 2H, J = 8.1 Hz, ArH), 3.79 (d, 4H, J = 6.1 Hz, –CH_2Cl), 3.67 (d, 4H, J = 6.2 Hz, –NCH_2–), 3.03 (s, 6H, –$N(CH_3)_2$); IR (KBr) ν (cm^{-1}): 2923, 2855, 1644, 1605, 1559, 1521, 1440, 1360, 1170, 810, 728; HRMS (ESI) calcd. for $C_{21}H_{24}Cl_2N_2O$ $[M+H]^+$: 391.1338, found: 391.1349.

Cytotoxicity evaluation

All reagents were handled in a sterile fume hood. RPMI 1640 medium, fetal bovine serum (FBS) and HEPES were purchased from Gibco; phosphate-buffered saline pH 7.4 (PBS) and trypsin–EDTA (0.25% trypsin and 1 mM Na_4(EDTA)) were obtained from Sigma–Aldrich. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was a Genview product. The growth medium (RPMI 1640 medium with 10% FBS, 100 U/mL penicillin, and 100 μg/L streptomycin), HEPES, and MTT were stored at 4°C, while trypsin–EDTA and FBS were stored frozen at −20°C and thawed before use; PBS was stored at r.t.

The human cancer cell line HepG2 (liver cells) was obtained from the Fourth Military Medical University; the K562 cell line (human leukemia cells) was obtained from BeiJing DingGuo ChangSheng Biotechnology Co., Ltd. Cells were cultured in 50-cm^2 culture flasks (Corning) using RPMI-1640 medium supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The culture was maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The cells were transferred to a new flask every 2 days and treated with trypsin–EDTA to detach them from the flask.

Cells were counted under a microscope using a hemacytometer (Hausser Scientific, 0.100 mm deep). Cell solutions were diluted with growth medium to a concentration of 1×10^5 cells/mL and transferred to a 96-well plate, by filling the wells in columns 2–10 (Fig. 4) with 100 μL (1×10^4 cells). Growth medium (100 μL) was added to column 11 as a blank. To each of the perimeter wells was added culture medium (100 μL),

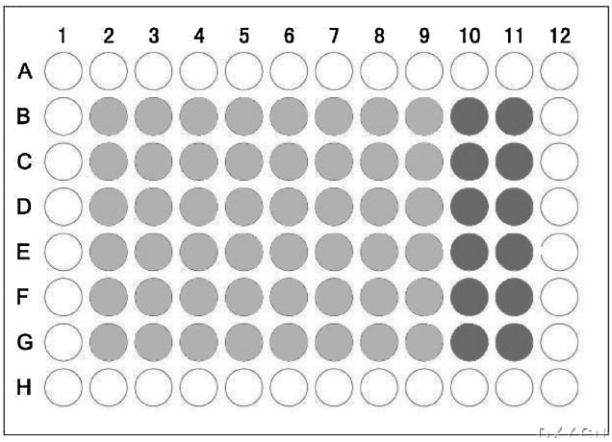


Figure 4. A 96-well plate showing the shaded wells in columns 2–11 used for the experiments.

and the plate was then incubated at 37°C for 24 h. A culture medium-diluted solution (100 μL) of the test compounds in DMSO (Grenier, Germany) was added into the wells in columns 2 (lowest) to 9 (highest), respectively, to obtain the required quantitative concentrations of 256, 64, etc. down to 2 μg/mL; the culture medium (100 μL) was also added to the wells in columns 10 and 11, and the plate was incubated at 37°C for 72 h.

A modified procedure of Mosmann [38, 39] was used for the MTT assays. An MTT solution in PBS (20 μL, 2.5 mg/mL) was added to each well of the plate, which was then incubated for 4 h, by which time a purple precipitate of formazan formed at the bottom of certain wells. The contents of each well were carefully aspirated off to leave the formazan, and then 150 μL DMSO was added and mixed thoroughly to dissolve the dark-blue crystals. The plates were read on a SpectraMax 190 microplate reader (Molecular Devices Inc., USA) at 490 nm within 1 h of DMSO addition. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%. The experiment was repeated three times.

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