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Synthesis, biological evaluation and molecular docking studies of amide-coupled benzoic nitrogen mustard derivatives as potential antitumor agents

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ABSTRACT

A series of amide-coupled benzoic nitrogen mustard derivatives as potential EGFR and HER-2 kinase inhibitors were synthesized and reported for the first time. Some of them exhibited significant EGFR and HER-2 inhibitory activity. Of all the studied compounds, compounds **5b** and **5t** exhibited the most potent inhibitory activity, which was comparable to the positive control erlotinib. Docking simulation was performed to position compounds **5b** and **5t** into the EGFR active site to determine the probable binding model. Antiproliferative assay results indicated that some of the benzoic nitrogen mustard derivatives possessed high antiproliferative activity against MCF-7. In particular, compounds **5b** and **5t** with potent inhibitory activity in tumor growth inhibition may function as potential antitumor agents.

1. Introduction

Receptor protein tyrosine kinases play a key role in signal transduction pathways that regulate cell division and differentiation. Among the growth factor receptor kinases that have been identified as being important in cancer is epidermal growth factor receptor (EGFR) kinase (also known as erb-B1 or HER-1) and the related human epidermal growth factor receptor HER-2 (also known as erbB-2). Deregulation of growth-factor signaling due to hyperactivation of the ErbB receptors (primarily EGFR and HER-2) is seen in several cancer types.[1,2] Activation of EGFR may be because of overexpression, mutations resulting in constitutive activation, or autocrine expression of ligand. In contrast, activation of HER-2 occurs mainly by overexpression, which leads to spontaneous homodimerization and activation of downstream signaling events in a ligand-independent manner.[3,4] The role of EGFR and HER-2 has been most thoroughly studied in breast cancer, where it is overexpressed in 25–30% of cases and is correlated with a poor prognosis. EGFR and HER-2 overexpression is also seen in ovarian cancer,[5] lung cancer (especially lung adenocarcinomas)[6–8] and in hormone-refractory prostate cancer.[9] Compounds that inhibit the kinase activity of EGFR and/or HER-2 after binding of its cognate ligand are of potential interest as new therapeutic antitumor agents.[10,11]

Nitrogen mustards (chlorambucil, melphalan) are widely used in the treatment of a variety of cancer.[12–14] But because of the high reactivity of nitrogen mustards, they are chemically unstable and produce many unwanted side effects including bone marrow toxicity and genotoxicity.[15,16] Moreover, a good many nitrogen mustards are typical bifunctional DNA-alkylating agents, nevertheless, their EGFR and/or HER-2 inhibitory activity has been less widely documented.[17] However, benzoic nitrogen mustard possessing relatively low toxicity is one of the earliest antitumor drugs in antibody-directed enzyme prodrug therapy (ADEPT).[18] It is reported that benzoic nitrogen mustard derivatives (e.g., **CMDA**, **CJS 1050**) (Fig. 1) possess favorable anticancer activity.[19]

In view of these above mentioned facts and an attempt to achieve new potent antitumor agents with good bioavailability and low toxicity and develop new benzoic nitrogen mustard derivatives as potential inhibitors of EGFR and HER-2, herein, we first describe the synthesis and the SAR of a series of amide-coupled benzoic nitrogen mustard derivatives. Biological evaluation indicated that some of the synthesized compounds exhibited favorable EGFR and HER-2 inhibitory activity and displayed significant inhibitory activity in tumor growth inhibition. Docking simulations were performed using the X-ray crystallographic structure of the EGFR in complex with an inhibitor to explore the binding modes of these compounds at the active site. To the best of our knowledge, the synthesis and biological evaluation of these compounds have not been reported so far.

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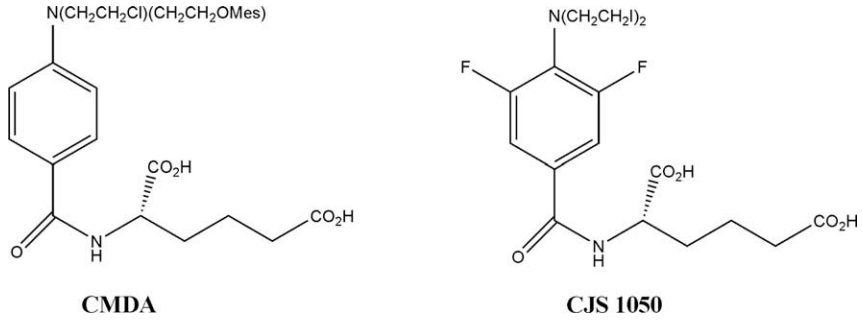
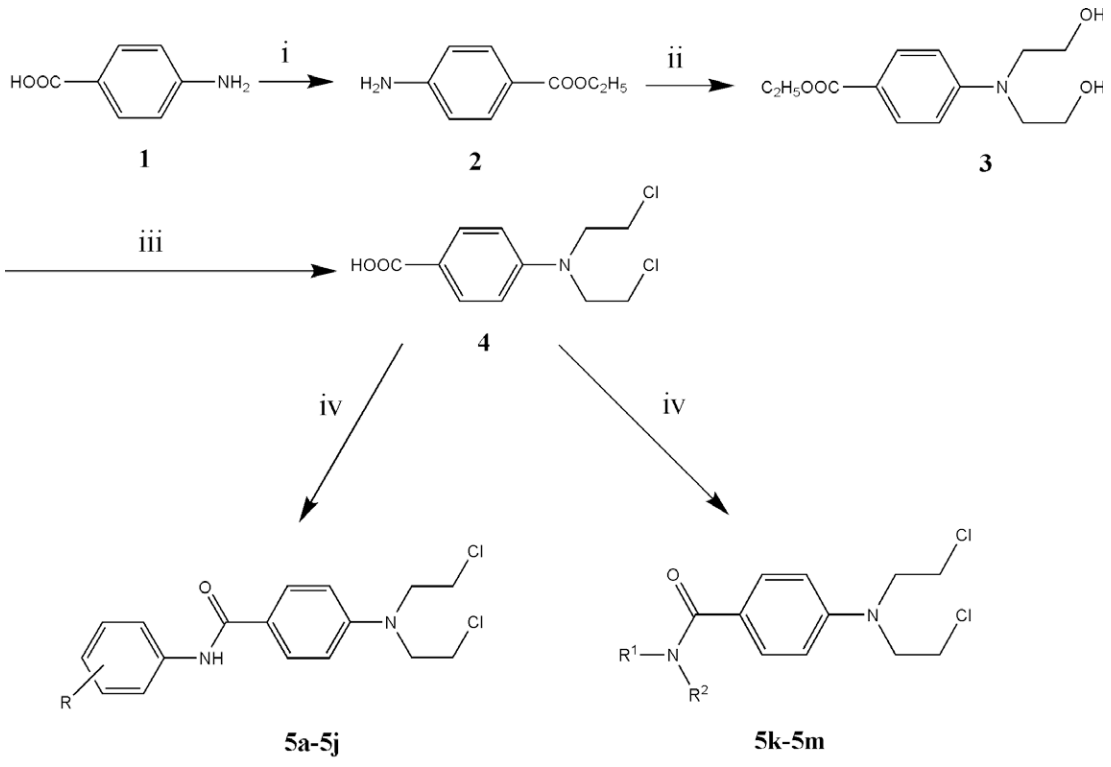


Figure 1. The chemical structures of **CMDA** and **CJS 1050**.

2. Results and discussion

2.1. Chemistry

The synthetic route for the new amide-coupled benzoic nitrogen mustard derivatives **5a–t** is outlined in Schemes 1 and 2. The synthesis of these nitrogen mustard derivatives started from 4-(bis(2-chloroethyl)amino)benzoic acid (**4**). Compound **4** was prepared according to modified procedures of Taylor et al.[20] Condensation of 4-aminobenzoic acid (**1**) with anhydrous ethanol afforded ethyl 4-aminobenzoate (**2**). Treatment of compound **2** with ethylene oxide in acetic acid aqueous solution at room temperature gave ethyl 4-(bis(2-hydroxyethyl)amino)benzoate (**3**). Reaction of compound **3** with thionyl chloride in benzene under reflux for 3 h, and then acidation with concentrated HCl under reflux for 3 h provided compound **4**. Target compounds **5a–t** were synthesized by the coupling reaction between compound **4** and corresponding arylamines or fatty amines or 3-morpholinopropan-1-amine using *N*,*N*′-2 dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (4-DMAP) in anhydrous dichloromethane.



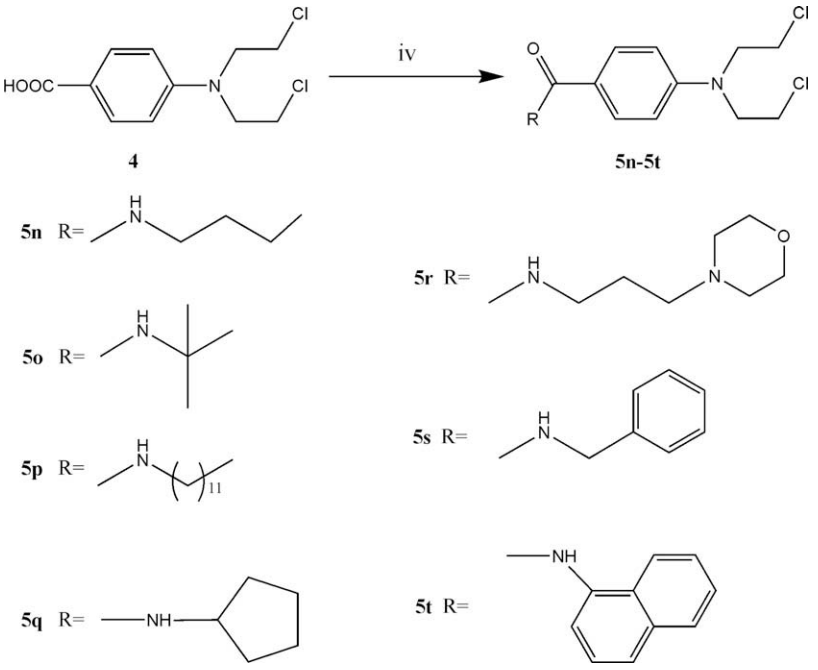
5a R= para-CH_3
5b R= para-CH_3O
5c R= para-$CH(CH_3)_2$
5d R= ortho-F
5e R= ortho-Cl
5f R= ortho-Br
5g R= para-F
5h R= para-Cl
5i R= para-Br
5j R= para-NO_2

5k R^1= n-propyl, R^2= n-propyl

5l R^1= n-butyl, R^2= n-butyl

5m R^1= C_6H_5 , R^2= CH_3

Scheme 1. Synthesis of compounds **5a–5m**. Reagents and conditions: (i) H_2SO_4 (concd), ethanol, reflux, 6 h; (ii) ethylene oxide, 25% aqueous acetic acid, rt, 24 h; (iii) (1) $SOCl_2$, benzene, reflux, 3 h; (2) HCl (concd), reflux, 3 h; (iv) DCC/4-DMAP, DCM, rt or reflux, 24 h.



Scheme 2. Synthesis of compounds **5n**–**5t**. Reagents and conditions: (iv) DCC/DMAP, DCM, rt or reflux, 24 h.

All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

2.2. Biological activity

All new synthesized compounds **5a–t** were evaluated for their ability to inhibit the autophosphorylation of EGFR and HER-2 kinases using a solid-phase ELISA assay. The results were shown in Table 1. For the studied compounds, we observed that the IC_{50} value for inhibition of HER-2 kinase is, in general, higher than that observed for EGFR kinase but have the same trends. This is possibly due, in part, to the fact that in the enzyme assays we used higher concentration of the purified HER-2 kinase than EGFR kinase. It is evident that there is also a reasonable correlation between the EGFR and HER-2 inhibitory activities; thus, this is not surprising in view of the high sequence homology of the catalytic domains of these two kinases. As shown in Table 1, some of nitrogen mustard derivatives displayed significant EGFR and HER-2 inhibitory activity. Of all the studied compounds, compounds **5b** and **5t** exhibited the most potent inhibitory activity (as for **5b**, IC_{50} = 0.08 μM for EGFR and IC_{50} = 0.41 μM for HER-2; as for **5t**, IC_{50} = 0.09 μM for EGFR and IC_{50} = 0.35 μM for HER-2), which was comparable to the positive control erlotinib (IC_{50} = 0.02 μM for EGFR).

Table 1
Inhibition (IC_{50}) of EGFR and HER-2 kinases and inhibition (IC_{50}) of cell proliferation

Compounds	Enzyme assays (IC_{50}, μM)		MCF-7
	EGFR	HER-2	(IC_{50}, μM)
5a	0.58	1.88	0.18 ± 0.03
5b	0.08	0.41	0.05 ± 0.02
5c	0.75	2.32	0.27 ± 0.04
5d	11.57	15.39	4.52 ± 0.28
5e	9.46	12.87	4.08 ± 0.39
5f	7.82	9.95	3.45 ± 0.41
5g	1.72	3.89	2.24 ± 0.18
5h	1.49	3.33	1.71 ± 0.22
5i	0.94	2.58	0.62 ± 0.14
5j	19.26	27.19	11.07 ± 1.13
5k	29.83	39.64	15.57 ± 2.32
5l	31.18	44.51	18.45 ± 2.27
5m	14.72	22.75	8.19 ± 0.56
5n	22.81	30.06	14.68 ± 1.15
5o	31.43	46.10	17.73 ± 1.21
5p	>50	>50	44.2 ± 3.55
5q	35.29	48.35	22.75 ± 2.93
5r	>50	>50	36.5 ± 3.22
5s	17.51	25.87	9.82 ± 1.37
5t	0.09	0.35	0.06 ± 0.02
Erlotinib	0.02	0.16	0.02 ± 0.004

Structure–activity relationships in these nitrogen mustard derivatives demonstrated that compounds containing arylamine moiety (e.g., **5a**–**j**, **5m**, **5s**, **5t**) showed better inhibitory activity than those containing fatty amine moiety (e.g., **5k**, 5**l**, **5n**–**q**). Moreover, compounds with electron-withdrawing groups on the *ortho* or *para* position of benzene ring (e.g., **5d**–**f**, **5g**–**j**) displayed less inhibitory activity than those with electron-donating groups on the *para* position (e.g., **5a**–**c**). Meanwhile, a comparison of the substitution on benzene ring demonstrated that *para* position-halogen-substituted derivatives (e.g., **5g**–**i**) have more potent EGFR inhibitory activity than that of *ortho* position-halogen-substituted derivatives (e.g., **5d**–**f**), and the potency order is F < Cl < Br. Besides, introduction of a heterocycle, as for compound **5r**, resulted in inactive inhibitory activity with IC_{50} values of more than 50 μM. Furthermore, variation of the aromatic ring moiety was also explored. Compared with compound **5b**, compound **5t** bearing naphthalen-1-amine moiety was compatible with the most potent inhibitory activity. That is to say, compounds **5b** and **5t** exhibited the most potent EGFR inhibitory activity with IC_{50} of 0.08 and 0.09 μM, respectively, which was comparable to the positive control.

In an effort to elucidate the structure–activity relationships observed at the EGFR and guide further SAR studies, molecular docking of the most potent inhibitors **5b** and **5t** into ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb). The binding models of compounds **5b** and **5t** and EGFR are depicted in Figures 2 and 3, respec-

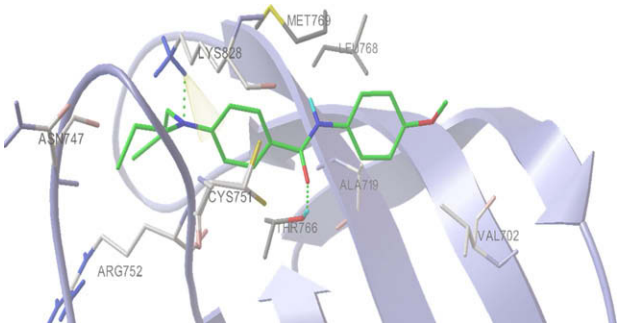


Figure 2. Molecular docking modeling of compound **5b** with EGFR kinase: The carbonyl group of **5b** forms hydrogen bond (O–H···O: 2.073 Å, 159.6°) with hydroxyl group of Thr 766, and the nitrogen atom of 4-(bis(2-chloroethyl)amino) group of **5b** also forms hydrogen bond (N–H···N: 1.967 Å, 134.8°) with amino hydrogen of Lys 828, and there is a π-cation interaction between the benzene ring of 4-(bis(2-chloroethyl)amino)benzoic acid moiety of **5b** and Lys 828.

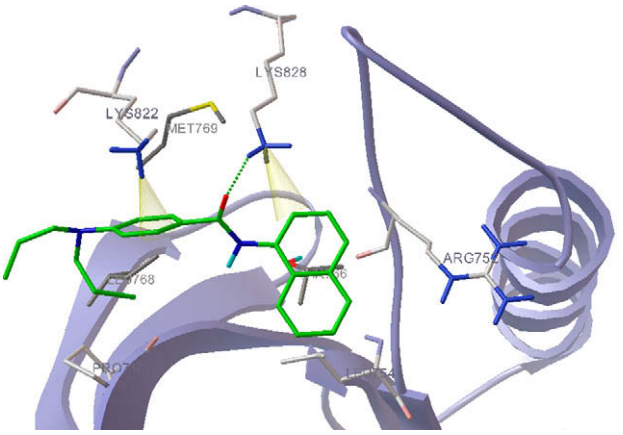


Figure 3. Molecular docking modeling of compound **5t** with EGFR kinase: The carbonyl group of **5t** forms hydrogen bond (N–H···O: 2.179 Å, 155.6°) with amino hydrogen of Lys 828. There is one π-cation interaction between the naphthalene ring of **5t** and Lys 828, and there is the other π-cation interaction between the benzene ring of 4-(bis(2-chloroethyl)amino)benzoic acid moiety of **5t** and Lys 822.

tively. In the binding model, compound **5b** is nicely bound to the region with the carbonyl group project toward the hydroxyl group of Thr 766, with the carbonyl group forming a more optimal H-bond (O–H···O: 2.073 Å, 159.6°) interaction, and nitrogen atom of 4-(bis(2-chloroethyl)amino) group of **5b** also forms hydrogen bond (N–H···N: 1.967 Å, 134.8°) with amino hydrogen of Lys 828. The modeling also suggested that there is a π-cation interaction between the benzene ring of 4-(bis(2-chloroethyl)amino)benzoic acid moiety of **5b** and Lys 828. The π-cation interaction is a noncovalent molecular interaction between the face of an electron-rich π system (e.g., benzene, ethylene) with an adjacent cation. The π-cation interaction energies are of the same order of magnitude as hydrogen bonds or salt bridges and play an important role in stabilizing the three-dimensional structure of a protein.[21,22] In the binding model, compound **5t** is nicely bound to the region with the carbonyl group project toward the amino group of Lys 828, with the carbonyl group forming a more optimal H-bond (N–H···O: 2.179 Å, 155.6°) interaction. Furthermore, there is one π-cation interaction between the naphthalene ring of **5t** and Lys 828, and there is the other π-cation interaction between the benzene ring of 4-(bis(2-chloroethyl)amino)benzoic acid moiety of **5t** and Lys 822. The enzyme assay data and the molecular docking results indicated that compounds **5b** and **5t** were potential inhibitors of EGFR.

Encouraged by these promising results, the in vitro antiproliferative activity of compounds **5a–t** was evaluated on a panel of one human tumor cell line (MCF-7), which over expresses EGFR and, to a less extent HER-2. To our delight, compounds **5a–c**, **5i** and **5t**, which have potent inhibitory activity of EGFR and HER-2 showed high antiproliferative activity against MCF-7 with IC_{50} ranging from 0.05 μM to 0.62 μM, which indicated that these amide-coupled benzoic nitrogen mustard derivatives were potent inhibitor of EGFR and HER-2 as antitumor agents. In particular, compounds **5b** and **5t** displayed significant inhibitory activity in tumor growth inhibition and exhibited favorable EGFR and HER-2 inhibitory activity.

3. Conclusion

In summary, a series of new amide-coupled benzoic nitrogen mustard derivatives were synthesized, some of which displayed potent EGFR and HER-2 inhibitory. Of all the studied compounds, compounds **5b** and **5t** exhibited the most potent inhibitory activity (as for **5b**, IC_{50} = 0.08 μM for EGFR and IC_{50} = 0.41 μM for HER-2; as for **5t**, IC_{50} = 0.09 μM for EGFR and IC_{50} = 0.35 μM for HER-2), which was comparable to the positive control erlotinib. Docking simulation was performed to position compounds **5b** and **5t** into the EGFR active site to determine the probable binding model. Antiproliferative assay results indicated that some of the benzoic nitrogen mustard derivatives possessed high antiproliferative activity against MCF-7. In particular, compounds **5b** and **5t** with potent inhibitory activity in tumor growth inhibition would be potential antitumor agents.

4. Experimental

4.1. Chemistry general

Separation of the compounds by column chromatography was carried out with Silica Gel 60 (200–300 mesh ASTM, E. Merck). The quantity of silica gel used was 50–100 times the weight charged on the column. Then, the eluates were monitored using thin-layer chromatography (TLC). TLC was carried out on silica GF254 plates (Qingdao Haiyang Chemical Co., Ltd, China). All the melting points were determined on a WRS-1B digital melting point apparatus and are uncorrected. ESI mass spectra were recorded on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were recorded on a Bruker DRX 500 or DPX 300 model Spectrometer in chloroform-*d* ($CDCl_3$) and tetramethylsilane (TMS) was used as an internal standard. Elemental analyses were performed on a CHN–O-Rapid instrument and were within ±0.4% of the theoretical values. All chemicals were used as received without further purification unless otherwise stated.

4.2. General procedure for the preparation of target compounds 5a–t

Compound **2**: To a solution of compound **1** (17 g) in ethanol (50 mL) was added dropwise concentrated sulfuric acid (12 mL). The mixture was heated under reflux for 6 h. The resulting solution was made neutral by addition of concentrated ammonia water. The precipitation was collected by filtration and then washed with water and subsequently dried to afford compound **2** (yield: 90.0%).

Compound **3**: To a chilled suspension of compound **2** (15.3 g, 93 mmol) prepared above in 25% aqueous acetic acid (120 mL) was added ethylene oxide (50 mL, 1.0 mol) with stirring. The reaction was left to stir for 24 h at room temperature to yield a clear solution. The solution was made basic by addition of concentrated ammonia water and extracted with ethyl acetate (200 mL × 3).

Pooled organic extracts were dried over anhydrous $MgSO_4$, filtered and excess solvent removed under reduced pressure to yield the crude product. Flash chromatography (ethyl acetate/petroleum ether = 1:2) gave the desired compound **3** (yield: 84.5%).

Compound **4**: Compound **3** (6.9 g, 27 mmol) prepared above was added to benzene (80 mL) containing thionyl chloride (10 mL, 137 mmol). The solution was heated at reflux for 3 h and after cooling to room temperature, the solvent was removed under reduced pressure to yield yellow oil. To the resulting oil, concentrated HCl (55 mL) was added and the solution heated at reflux for 3 h. After the reaction mixture cooled overnight, the precipitate was filtered, washed with cold 50% aqueous ethanol to obtain compound **4** (yield: 45.0%).

To a stirred solution of **4** (0.26 g, 1 mmol) in anhydrous dichloromethane (12 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (DCC, 0.21 g, 1 mmol), corresponding arylamines or fatty amines or 3-morpholinopropan-1-amine and 4-dimethylaminopyridine (4-DMAP, 0.12 g, 0.2 mmol). The reaction mixture was left stirring at room temperature (for target compounds **5a–c**, **5k**, **5l** and **5n–q**) or at reflux (for target compounds **5d–j**, **5m**, and **5r–t**) for 24 h. The resulting mixture was filtered, washed with dichloromethane, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified with a silica gel column and was eluted with ethyl acetate/petroleum ether = 1:3–1:8 to afford target compounds **5a–t**.

4.2.1. 4-(Bis(2-chloroethyl)amino)-*N*-*p*-tolylbenzamide (5a)

White powder. Yield: 86%; mp: 130–132 °C; ^{1}H NMR (300 MHz, $CDCl_3$): 2.32 (s, 3H); 3.66 (t, *J* = 7.0 Hz, 4H); 3.80 (t, *J* = 7.1 Hz, 4H); 6.70 (d, *J* = 8.9 Hz, 2H); 7.15 (d, *J* = 8.9 Hz, 2H); 7.50 (d, *J* = 8.4 Hz, 2H); 7.71 (s, 1H); 7.79 (d, *J* = 8.9 Hz, 2H). MS (ESI): 352.3 ($C_{18}H_{20}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}Cl_2N_2O$: C, 61.55; H, 5.74; N, 7.97. Found: C, 61.70; H, 5.60; N, 7.81.

4.2.2. 4-(Bis(2-chloroethyl)amino)-*N*-(4-methoxyphenyl) benzamide (5b)

White powder. Yield: 88%; mp: 140–141 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.67 (t, *J* = 6.4 Hz, 4H); 3.79–3.83 (m, 7H); 6.72 (d, *J* = 7.2 Hz, 2H); 6.90 (d, *J* = 7.2 Hz, 2H); 7.52 (d, *J* = 9.0 Hz, 2H); 7.59 (s, 1H); 7.79 (d, *J* = 9.0 Hz, 2H). MS (ESI): 368.3 ($C_{18}H_{20}Cl_2N_2O_2$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}Cl_2N_2O_2$: C, 58.86; H, 5.49; N, 7.63. Found: C, 58.67; H, 5.68; N, 7.81.

4.2.3. 4-(Bis(2-chloroethyl)amino)-*N*-(4-isopropylphenyl) benzamide (5c)

White powder. Yield: 84%; mp: 130–131 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.22 (d, *J* = 6.9 Hz, 6H); 2.84–2.89 (m, 1H); 3.67 (t, *J* = 6.6 Hz, 4H); 3.81 (t, *J* = 6.6 Hz, 4H); 6.73 (d, *J* = 9.0 Hz, 2H); 7.22 (d, *J* = 9.0 Hz, 2H); 7.53 (d, *J* = 9.0 Hz, 2H); 7.63 (s, 1H); 7.80 (d, *J* = 9.1 Hz, 2H). MS (ESI): 380.3 ($C_{20}H_{24}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{24}Cl_2N_2O$: C, 63.33; H, 6.38; N, 7.39. Found: C, 63.42; H, 6.49; N, 7.21.

4.2.4. 4-(Bis(2-chloroethyl)amino)-*N*-(2-fluorophenyl)benzamide (5d)

White powder. Yield: 65%; mp: 80–82 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.68 (t, *J* = 6.8 Hz, 4H); 3.83 (t, *J* = 6.8 Hz, 4H); 6.75 (d, *J* = 9.0 Hz, 2H); 7.04–7.19 (m, 3H); 7.83 (d, *J* = 8.9 Hz, 2H); 7.96 (s, 1H); 8.45–8.51 (m, 1H). MS (ESI): 356.2 ($C_{17}H_{17}Cl_2FN_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}Cl_2FN_2O$: C, 57.48; H, 4.82; N, 7.89. Found: C, 57.63; H, 4.70; N, 7.71.

4.2.5. 4-(Bis(2-chloroethyl)amino)-*N*-(2-chlorophenyl)benzamide (5e)

White powder. Yield: 60%; mp: 111–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.66 (t, *J* = 6.8 Hz, 4H); 3.81 (t, *J* = 6.8 Hz, 4H); 6.73 (d, *J* = 9.0 Hz, 2H); 7.06–7.17 (m, 3H); 7.84 (d, *J* = 8.9 Hz, 2H); 7.88 (s, 1H); 8.47–8.53 (m, 1H). MS (ESI): 372.7 ($C_{17}H_{17}Cl_3N_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}Cl_3N_2O$: C, 54.93; H, 4.61; N, 7.54. Found: C, 54.81; H, 4.75; N, 7.68.

4.2.6. 4-(Bis(2-chloroethyl)amino)-*N*-(2-bromophenyl)benzamide (5f)

White powder. Yield: 58%; mp: 104–106 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.68 (t, *J* = 6.6 Hz, 4H); 3.82 (t, *J* = 6.6 Hz, 4H); 6.74 (d, *J* = 9.0 Hz, 2H); 7.07–7.20 (m, 3H); 7.85 (d, *J* = 8.9 Hz, 2H); 7.91 (s, 1H); 8.46–8.53 (m, 1H). MS (ESI): 417.1 ($C_{17}H_{17}BrCl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}BrCl_2N_2O$: C, 49.07; H, 4.12; N, 6.73. Found: C, 49.26; H, 4.32; N, 6.54.

4.2.7. 4-(Bis(2-chloroethyl)amino)-*N*-(4-fluorophenyl)benzamide (5g)

White powder. Yield: 63%; mp: 125–127 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.66 (t, *J* = 6.6 Hz, 4H); 3.82 (t, *J* = 6.6 Hz, 4H); 6.65 (d, *J* = 8.9 Hz, 2H); 6.73 (d, *J* = 8.9 Hz, 1H); 7.06 (t, *J* = 8.7 Hz, 1H); 7.51–7.58 (m, 3H); 7.67 (s, 1H); 7.80 (d, *J* = 9.0 Hz, 1H). MS (ESI): 356.2 ($C_{17}H_{17}Cl_2FN_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}Cl_2FN_2O$: C, 57.48; H, 4.82; N, 7.89. Found: C, 57.69; H, 5.01; N, 7.68.

4.2.8. 4-(Bis(2-chloroethyl)amino)-*N*-(4-chlorophenyl)benzamide (5h)

White powder. Yield: 54%; mp: 116–118 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.66 (t, *J* = 6.8 Hz, 4H); 3.80 (t, *J* = 6.8 Hz, 4H); 6.68 (d, *J* = 8.9 Hz, 2H); 6.73 (d, *J* = 8.9 Hz, 1H); 7.02 (t, *J* = 8.9 Hz, 1H); 7.49–7.56 (m, 3H); 7.64 (s, 1H); 7.73 (d, *J* = 8.9 Hz, 1H). MS (ESI): 372.7 ($C_{17}H_{17}Cl_3N_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}Cl_3N_2O$: C, 54.93; H, 4.61; N, 7.54. Found: C, 54.72; H, 4.73; N, 7.41.

4.2.9. 4-(Bis(2-chloroethyl)amino)-*N*-(4-bromophenyl)benzamide (5i)

White powder. Yield: 51%; mp: 120–122 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.65 (t, *J* = 6.6 Hz, 4H); 3.80 (t, *J* = 6.6 Hz, 4H); 6.69 (d, *J* = 8.9 Hz, 2H); 6.77 (d, *J* = 8.9 Hz, 1H); 7.10 (t, *J* = 8.8 Hz, 1H); 7.55–7.62 (m, 3H); 7.66 (s, 1H); 7.83 (d, *J* = 9.0 Hz, 1H). MS (ESI): 417.1 ($C_{17}H_{17}BrCl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}BrCl_2N_2O$: C, 49.07; H, 4.12; N, 6.73. Found: C, 49.25; H, 4.31; N, 6.56.

4.2.10. 4-(Bis(2-chloroethyl)amino)-*N*-(4-nitrophenyl)benzamide (5j)

White powder. Yield: 49%; mp: 127–129 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.66 (t, *J* = 6.6 Hz, 4H); 3.82 (t, *J* = 6.6 Hz, 4H); 6.73 (d, *J* =9.0 Hz, 2H); 7.22 (d, *J* = 9.0 Hz, 2H); 7.68 (s, 1H); 7.72 (d, *J* = 9.0 Hz, 2H); 8.08 (d, *J* = 9.0 Hz, 2H). MS (ESI): 383.2 ($C_{17}H_{17}Cl_2N_3O_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}Cl_2N_3O_3$: C, 53.42; H, 4.48; N, 10.99. Found: C, 53.25; H, 4.27; N, 11.19.

4.2.11. 4-(Bis(2-chloroethyl)amino)-*N*,*N*-dipropylbenzamide (5k)

White powder. Yield: 78%; mp: 88–90 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 0.92 (t, *J* = 7.3 Hz, 6H); 1.36–1.46 (m, 4H); 3.56 (t, *J* = 6.6 Hz, 4H); 3.68 (t, *J* = 6.6 Hz, 4H); 3.78 (t, *J* = 6.6 Hz, 4H); 6.65 (d, *J* = 8.9 Hz, 2H); 7.68 (d, *J* = 8.9 Hz, 2H). MS (ESI): 346.3 ($C_{17}H_{26}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{26}Cl_2N_2O$: C, 59.13; H, 7.59; N, 8.11. Found: C, 59.02; H, 7.40; N, 8.32.

4.2.12. 4-(Bis(2-chloroethyl)amino)-*N*,*N*-dibutylbenzamide (5l)

White powder. Yield: 76%; mp: 91–93 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 0.95 (t, *J* = 7.3 Hz, 6H); 1.33–1.49 (m, 8H); 3.56 (t, *J* = 6.6 Hz, 4H); 3.68 (t, *J* = 6.6 Hz, 4H); 3.78 (t, *J* = 6.6 Hz, 4H); 6.66 (d, *J* = 9.0 Hz, 2H); 7.67 (d, *J* = 9.0 Hz, 2H). MS (ESI): 374.4 ($C_{19}H_{30}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{30}Cl_2N_2O$: C, 61.12; H, 8.10; N, 7.50. Found: C, 61.31; H, 7.97; N, 7.32.

4.2.13. 4-(Bis(2-chloroethyl)amino)-*N*-methyl-*N*-phenylbenzamide (5m)

White powder. Yield: 72%; mp: 107–109 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.16 (s, 3H); 3.66 (t, J = 7.0 Hz, 4H); 3.82 (t, J = 7.0 Hz, 4H); 6.73 (d, J = 8.8 Hz, 2H); 7.12 (t, J = 7.7 Hz, 1H); 7.36 (t, J = 8.3 Hz, 2H); 7.62 (d, J = 8.3 Hz, 2H); 7.80 (d, J = 8.8 Hz, 2H). MS (ESI): 352.3 ($C_{18}H_{20}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}Cl_2N_2O$: C, 61.55; H, 5.74; N, 7.97. Found: C, 61.38; H, 5.90; N, 7.83.

4.2.14. 4-(Bis(2-chloroethyl)amino)-*N*-butylbenzamide (5n)

White powder. Yield: 85%; mp: 52–54 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 0.95 (t, J = 7.3 Hz, 3H); 1.36–1.46 (m, 2H); 1.54–1.63 (m, 2H); 3.39–3.46 (m, 2H); 3.67 (t, J = 6.6 Hz, 4H); 3.77 (t, J = 6.6 Hz, 4H); 6.07 (s, 1H); 6.66 (d, J = 8.8 Hz, 2H); 7.69 (d, J = 8.8 Hz, 2H). MS (ESI): 318.3 ($C_{15}H_{22}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{22}Cl_2N_2O$: C, 56.79; H, 6.99; N, 8.83. Found: C, 56.68; H, 6.90; N, 8.94.

4.2.15. 4-(Bis(2-chloroethyl)amino)-*N*-*tert*-butylbenzamide (5o)

White powder. Yield: 80%; mp: 141–143 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.28 (s, 9 H); 3.63 (t, J = 6.6 Hz, 4H); 3.76 (t, J = 6.6 Hz, 4H); 5.83 (s, 1H); 6.68 (d, J = 8.9 Hz, 2H); 7.70 (d, J = 8.9 Hz, 2H); MS (ESI): 318.3 ($C_{15}H_{22}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{22}Cl_2N_2O$: C, 56.79; H, 6.99; N, 8.83. Found: C, 56.88; H, 6.72; N, 8.70.

4.2.16. 4-(Bis(2-chloroethyl)amino)-*N*-dodecylbenzamide (5p)

White powder. Yield: 75%; mp: 60–62 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 0.90 (t, J = 7.2 Hz, 3H); 1.19–1.31 (m, 18 H); 1.41–1.48 (m, 2 H); 3.12–3.16 (m, 2 H); 3.67 (t, J = 6.6 Hz, 4H); 3.81 (t, J = 6.7 Hz, 4H); 5.96 (s, 1H); 6.70 (d, J = 8.9 Hz, 2H); 7.71 (d, J = 8.8 Hz, 2H). MS (ESI): 430.5 ($C_{23}H_{38}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{38}Cl_2N_2O$: C, 64.32; H, 8.92; N, 6.52. Found: C, 64.51; H, 8.73; N, 6.34.

4.2.17. 4-(Bis(2-chloroethyl)amino)-*N*-cyclopentylbenzamide (5q)

White powder. Yield: 81%; mp: 84–86 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.67–1.69 (m, 4H); 1.70–1.73 (m, 4H); 3.64 (t, J = 6.8 Hz, 4H); 3.78 (t, J = 6.8 Hz, 4H); 4.33–4.45 (m, 1H); 5.90 (s, 1H); 6.67 (d, J = 8.9 Hz, 2H); 7.68 (d, J = 8.9 Hz, 2H). MS (ESI): 330.3 ($C_{16}H_{22}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{22}Cl_2N_2O$: C, 58.36; H, 6.73; N, 8.51. Found: C, 58.19; H, 6.58; N, 8.64.

4.2.18. 4-(Bis(2-chloroethyl)amino)-*N*-(3-morpholinopropyl) benzamide (5r)

White powder. Yield: 61%; mp: 93–95 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.68–1.71 (m, 2H); 2.33–2.36 (t, J = 6.6 Hz, 4H); 2.44–2.46 (m, 2H); 3.36–3.39 (m, 2H); 3.62–3.69 (m, 12H); 6.26 (s, 1H); 6.67 (d, J = 8.8 Hz, 2H); 7.37 (d, J = 8.8 Hz, 2H). MS (ESI): 389.3 ($C_{18}H_{27}Cl_2N_3O_2$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{27}Cl_2N_3O_2$: C, 55.67; H, 7.01; N, 10.82. Found: C, 55.79; H, 7.21; N, 10.63.

4.2.19. *N*-Benzyl-4-(bis(2-chloroethyl)amino)benzamide (5s)

White powder. Yield: 81%; mp: 105–107 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.64 (t, J = 6.6 Hz, 4H); 3.78 (t, J = 6.6 Hz, 4H); 4.64 (d, J = 5.7 Hz, 2H); 6.26 (s, 1H); 6.67 (d, J = 9.0 Hz, 2H); 7.30–7.36(m, 5H); 7.72 (d, J = 9.0 Hz, 2H). MS (ESI): 352.3 ($C_{18}H_{20}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}Cl_2N_2O$: C, 61.55; H, 5.74; N, 7.97. Found: C, 61.36; H, 5.91; N, 7.80.

4.2.20. 4-(Bis(2-chloroethyl)amino)-*N*-(1-naphthyl)benzamide (5t)

White powder. Yield: 72%; mp: 107–109 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.66 (t, J = 6.6 Hz, 4H); 3.83 (t, J = 6.6 Hz, 4H); 6.74 (d, J = 8.8 Hz, 2H); 7.35 (d, J = 8.8 Hz, 2H); 7.56–7.61 (m, 3H); 7.86 (s, 1H); 7.90–7.94 (d, J = 8.0 Hz, 2H); 8.04 (d, J = 8.0 Hz, 2H). MS (ESI): 388.3 ($C_{21}H_{20}Cl_2N_2O$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{20}Cl_2N_2O$: C, 65.12; H, 5.20; N, 7.23. Found: C, 65.29; H, 5.35; N, 7.12.

4.3. Preparation, purification of HER-2 and EGFR and inhibitory assay

A 1.7 Kb cDNA encoded for human HER-2 cytoplasmic domain (HER-2-CD, amino acids 676–1245) and 1.6 kb cDNA encoded for the EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186) were cloned into baculoviral expression vectors pBlueBacHis2B and pFASTBacHTc (Huakang Company China), separately. A sequence that encodes $(His)_6$ was located at the 5′ upstream to the HER-2 and EGFR sequences. Sf-9 cells were infected for 3 days for protein expression. Sf-9 cell pellets were solubilized at 0 °C in a buffer at pH 7.4 containing 50 mM HEPES, 10 mM NaCl, 1% Triton, 10 μM ammonium molybdate, 100 μM sodium vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 16 μg/mL benzamidine HCl for 20 min followed by 20 min centrifugation. Crude extract supernatant was passed through an equilibrated Ni-NTA superflow packed column and washed with 10 mM and then 100 mM imidazole to remove nonspecifically bound material. Histidinetagged proteins were eluted with 250 and 500 mM imidazole and dialyzed against 50 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin for 2 h. The entire purification procedure was performed at 4 °C or on ice.[23]

Both EGFR and HER-2 kinase assays were set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Target compounds **5a–t** were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. In each well, 10 μL of compound was incubated with 10 μL (12.5 ng for HER-2 or 5 ng for EGFR) of recombinant enzyme (1:80 dilution in 100 mM HEPES) for 10 min at room temperature. Then, 10 μL of 5 × buffer (containing 20 mM HEPES, 2 mM $MnCl_2$, 100 μM Na_3VO_4, and 1 mM DTT) and 20 μL of 0.1 mM ATP-50 mM $MgCl_2$ was added for 1 h. Positive and negative controls were included in each plate by incubation of enzyme with or without ATP-$MgCl_2$. At the end of incubation, liquid was aspirated, and plates were washed three times with wash buffer. A 75 μL (400 ng) sample of europiumlabeled anti-phosphotyrosine antibody was added to each well for another 1 h of incubation. After washing, enhancement solution was added and the signal was detected by Victor (Wallac Inc.) with excitation at 340 nm and emission at 615 nm. The percentage of autophosphorylation inhibition by the compounds was calculated using the following equation: 100% − [(negative control)/(positive control − negative control)]. The IC_{50} was obtained from curves of percentage inhibition with eight concentrations of compound. As the contaminants in the enzyme preparation are fairly low, the majority of the signal detected by the anti-phosphotyrosine antibody is from EGFR or HER-2.

4.4. Cell proliferation assay

The antiproliferative activity was determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of 7×10^3 cells/well in 96-well microtiter plates (Costar). After 24 h, exponentially growing cells were exposed to the indicated compounds at final concentrations ranging from 0.1 to 100 μg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (10 μL of 5 mg/mL MTT in PBS). After 4 h, 100 μL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 18 h; optical absorbance was measured at 570 nm on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages

with respect to untreated cells. IC_{50} values were determined from replicates of 6 wells from at least three independent experiments.

4.5. Molecular docking modeling

Molecular docking of compounds **5b** and **5t** into the three-dimensional EGFR complex structure (1M17.pdb, downloaded from the PDB) was carried out using the AutoDock software package (version 4.0) as implemented through the graphical user interface AutoDockTools (ADT 1.4.6).

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