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Synthesis and structure–activity relationships of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas as antitumor agents

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ABSTRACT

Two series of novel *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas (**1a**–**18a**; **1b**–**18b**) as potential EGFR and HER-2 kinase inhibitors have been discovered. These compounds displayed good EGFR and HER-2 inhibitory activity and the SARs are also been studied. Especially compound **7b** demonstrated significant EGFR and HER-2 inhibitory activity (IC_{50} = 0.08 μM for EGFR and IC_{50} = 0.35 μM for HER-2). Docking simulation was performed to position compound **7b** into the EGFR active site to determine the probable binding conformation and antiproliferative assay results indicating that these series of urea and thioureas own high antiproliferative activity against MCF-7. Above all, thiourea **7b** would be a potential anticancer agent deserves further research.

1. Introduction

Receptor protein tyrosine kinases play a key role in signal transduction pathways that regulate cell division and differentiation. Upon binding to their specific extracellular growth factors, receptor tyrosine kinases undergo dimerization and autophosphorylation, initiating a cascade of downstream signaling events. Many of the growth factor receptor proteins have intracellulardomains that function as tyrosine kinases, and it is with this that they effect signaling. The interaction of growth factors with these receptors is a necessary event in normal regulation of cell growth. However, under certain conditions, as a result of overexpression, mutation, or coexpression of the ligand and the receptor, these receptors can become hyperactivated; the result of this is uncontrolled cell proliferation.[1] 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.[2,3] 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.[4–6] 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. Overexpression occurs primarily as a result of gene amplification. EGFR and HER-2 overexpression is also seen in ovarian cancer,[7] lung cancer (especially lung adenocarcinomas),[8–10] and in hormone-refractory prostate cancer.[11] 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.[12,13]

The urea derivatives such as *N*-nitrosoureas, benzoylureas, thioureas represent one of the generally most useful classes of anticancer agents, with a wide range of activities against various leukemias and solid tumors. The urea and the thiourea derivatives play important role in anticancer agents because of their good inhibitory activity against Receptor tyrosine kinases (RTKs), protein tyrosine kinases (PTKs), and NADH oxidase, which play critical roles in many aspects of tumorigenesis.[14–16] For example, pyrazolopyrimidine ureas bound to the ATP binding site of KDR kinase, the amino thienopyrimidine core mimics the adenine of ATP in its interaction with the hinge region of KDR.[17] Hydrogen bonds are formed between the exocyclic amino group of pyrazolopyrimidine ureas and the backbone carbonyl of Glu 917 and the proximal ring nitrogen and the backbone N–H of Cys 919. Our interest in this area is to design and synthesize diverse active urea and thiourea derivatives for antitumor agents.[18–20] Since the tyrosine phosphorylation event catalyzed by EGFR or HER-2 propagates the signal for cell division and since deregulation of these kinases has been

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associated with disease, an inhibitor of this event may have potential therapeutic value. Since these two receptor kinases have a high sequence homology in their catalytic domains, in continuation of our earlier studies, we now first describe the synthesis and the SAR of two series of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas. These compounds are potent inhibitors of EGFR and HER-2, 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.

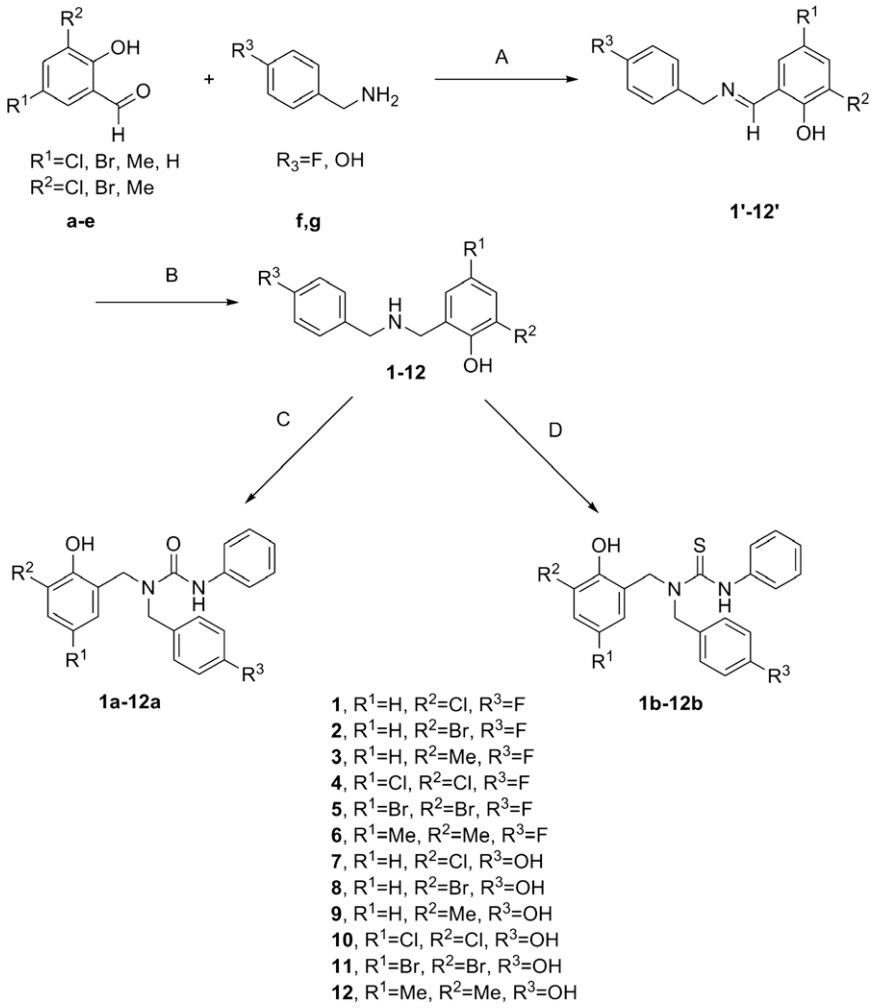
2. Results and discussion

2.1. Chemistry

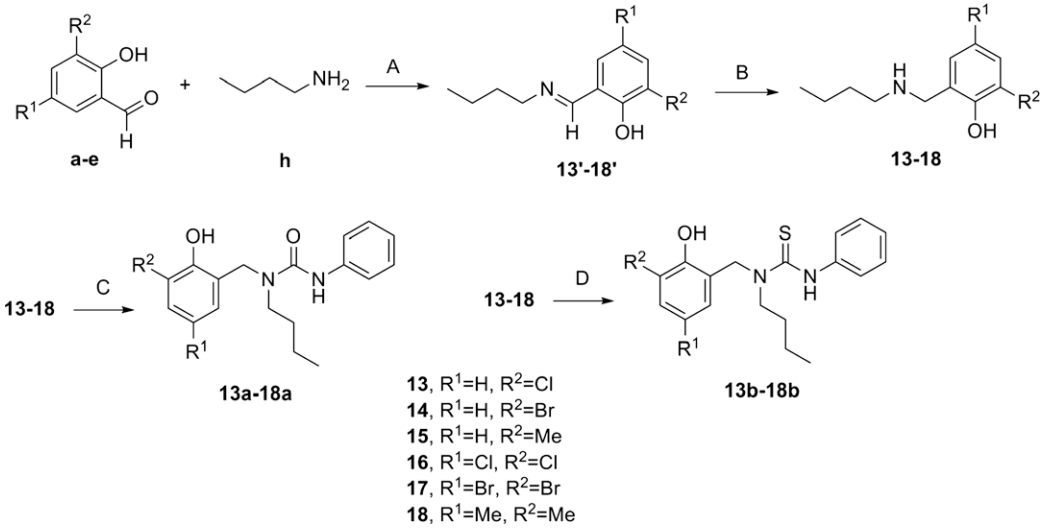
The route adapted for the synthesis of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas is outlined in Schemes 1 and 2. In our previous study, salicylaldehyde derivatives with halo-atoms in the R^1 and R^2 positions of aromatic ring showed potent biological activities, and the activity of methyl group in the R^1 and R^2 positions was low.[21] So we choose the salicylaldehyde with H, Cl, Br, and Me groups at R^1 and R^2 positions to prepare the corresponding Schiff bases. Without further purification, the secondary amines (**1**–**18**) were obtained from the corresponding Schiff bases after the reduction reactions with sodium borohydride in 60–90% yield.[22] In the next step, the secondary amines were condensed with phenylisocyanate or phenylisothiocyanate in chloroform as the solvent, affording the target compounds after 2–8 h for urea and thiouurea derivatives with electron-withdrawing substituent groups (**1a**–**12a** and **1b**–**12b**), and after 4 h for the electron-donating substituted derivatives (**13a**–**18a** and **13b**–**18b**). The reactions were monitored by thin layer chromatography (TLC) and the products were purified by recrystallization in ethanol. The yields were in the range of 55–95% for ureas and 47–88% for the thioureas. All the ureas and thioureas **1a–18a** and **1b–18b** were synthesized for the first time and all compounds were fully characterized by ^{1}H NMR, ESI MS and elemental analysis.

2.2. Biological activity and molecular modeling

The synthesized *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas were evaluated for their ability to inhibit the autophosphorylation of EGFR and HER-2 kinases using a solid-phase ELISA assay. The results were summarized in Table 1. For the given 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



Scheme 1. Synthesis of compounds **1a**–**12a** and **1b**–**12b**. Reagents and conditions: (A) ethanol, rt, 2 h; (B) ethanol, $NaBH_4$, reflux, 3–6 h; (C) phenylisocyanate, chloroform, reflux, 2–4 h; (D) phenylisothiocyanate, chloroform, reflux, 2–4 h.



Scheme 2. Synthesis of compounds **13a**–**18a** and **13b**–**18b**. Reagents and conditions: (A) ethanol, 60 °C, 4 h; (B) ethanol, $NaBH_4$, reflux, 8–10 h; (C) phenylisocyanate, chloroform, reflux, 4–6 h; (D) phenylisothiocyanate, chloroform, reflux, 4–6 h.

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

Compd	R^1	R^2	R^3	Enzyme assays IC_{50} (μM)		MCF-7 IC_{50} (μM)
				EGFR	HER-2	
1a	H	Cl	F	1.98	3.45	1.1 ± 0.3
2a	H	Br	F	2.24	5.17	1.4 ± 0.5
3a	H	Me	F	21.71	38.23	5.5 ± 2.3
4a	Cl	Cl	F	4.15	9.64	2.8 ± 1.3
5a	Br	Br	F	4.68	12.42	3.6 ± 1.4
6a	Me	Me	F	27.35	>50	6.6 ± 2.7
7a	H	Cl	OH	0.96	2.27	0.19 ± 0.04
8a	H	Br	OH	1.07	2.66	0.25 ± 0.06
9a	H	Me	OH	7.51	18.43	5.3 ± 2.6
10a	Cl	Cl	OH	1.86	2.21	1.1 ± 0.4
11a	Br	Br	OH	1.12	4.53	1.32 ± 0.46
12a	Me	Me	OH	9.26	16.12	8.5 ± 3.3
13a	H	Cl	—	32.12	>50	9.3 ± 2.7
14a	H	Br	—	36.88	>50	9.1 ± 2.9
15a	H	Me	—	>50	>50	52 ± 24
16a	Cl	Cl	—	>50	>50	30 ± 9
17a	Br	Br	—	>50	>50	35 ± 13
18a	Me	Me	—	>50	>50	26 ± 8
1b	H	Cl	F	0.67	1.41	0.52 ± 0.45
2b	H	Br	F	0.98	1.87	0.73 ± 0.6
3b	H	Me	F	8.54	15.66	3.8 ± 1.3
4b	Cl	Cl	F	1.34	6.49	1.1 ± 0.4
5b	Br	Br	F	1.76	6.73	1.7 ± 0.5
6b	Me	Me	F	19.41	37.82	10.5 ± 4.4
7b	H	Cl	OH	0.08	0.35	0.03 ± 0.01
8b	H	Br	OH	0.12	0.66	0.06 ± 0.04
9b	H	Me	OH	3.46	7.13	1.2 ± 0.6
10b	Cl	Cl	OH	0.56	1.23	0.22 ± 0.21
11b	Br	Br	OH	0.87	2.24	0.36 ± 0.32
12b	Me	Me	OH	5.83	9.47	2.6 ± 1.0
13b	H	Cl	—	18.12	32.54	7.4 ± 2.6
14b	H	Br	—	22.04	39.51	14.2 ± 5.1
15b	H	Me	—	43.21	>50	23.6 ± 7.41
16b	Cl	Cl	—	24.65	39.78	12.8 ± 3.7
17b	Br	Br	—	31.94	>50	16.7 ± 6.3
18b	Me	Me	—	>50	>50	34.1 ± 11.1
Erlotinib				0.02	0.12	0.01 ± 0.003

the high sequence homology of the catalytic domains of these two kinases.

A number of synthesized compounds displayed potent EGFR and HER-2 inhibitory activity. *N*-(5-Chloro-2-hydroxybenzyl)-*N*-(4-hydroxybenzyl)-*N*′-phenylthiourea (**7b**) showed the most potent inhibitory activity (IC_{50} = 0.08 μM for EGFR and IC_{50} = 0.35 μM for HER-2), and comparable to the positive control erlotinib (MIC IC_{50} = 0.02 μM). In the all ureas and thioureas, thioureas (**1b**–**18b**) displayed potent inhibitory activity than ureas (**1a**–**18a**), it suggests that the external N–H of the thiourea has a better hydrogen bond interaction with the protein than the urea.

Structure–activity relationships in these new compounds demonstrated that compounds containing a R^1 and/or R^2 = Br or Cl are consistently more potent than those with a methyl group. This trend was observed in all the series of compounds used herein whether they were ureas or thioureas. Many compounds with $R^1 = R^2$ or R^1 = H (e.g., **1b** and **5b**, **2b** and **4b**) shown IC_{50} in the 1 μM range, indicating little tolerance of bulkiness in the hydrophobic pocket of the ATP site of EGFR (and, presumably, HER-2). Hydroxyl derivatives at R^3 position are more potent then corresponding fluorine derivatives, so the increased lipophilicity of fluorine derivatives at R^3 position may be detrimental to the inhibitory activity. Ureas and thioureas with alkyl group at N-2 (**13a**–**18a** and **13b**–**18b**) displayed weaker inhibitory activity (most >50 μM) than others, we surmised that the alkyl chain group in the urea and thioureas led to decrease in EGFR and HER-2 inhibitory activity due to the ability of the alkyl chain group to impose a torsion angle between the plane of the aromatic urea bicycle and that of the ureido function of the resulting molecules. This may lead to a less planar structure, a geometry that may hinder its binding in the ATP pocket of EGFR (and, presumably, HER-2).

To help understand the SARs observed at the EGFR and guide further SAR studies, molecular docking of the most potent inhibitor **7b** into ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb). The binding model of compound **7b** and EGFR is depicted in Figure 1. In the binding model, thiourea **7b** is nicely bound to the region with the urea N–H groups of **7b** project toward the side chain carbonyl group of Leu 768, with the external N–H forming a more optimal H-bond interaction; the hydroxyl group of **7b** also forms hydrogen bond with amino hydrogen of Gln767, while the corresponding urea **7a** can not bound nicely. There is only carbonyl group bond with the amine group of Lys828 and a π-cation interaction between benzyl ring of compound **7a** and Lys828 (Fig. 2). These were supported by EGFR potency difference between ureas (**1a–18a**) and thioureas (**1b–18b**). The modeling also suggested that there is a π-cation interaction between R^3-benzyl ring of compound **7b** and Lys828, π-cation interaction energies are of the same order of magnitude as hydrogen bonds or salt bridges and play an

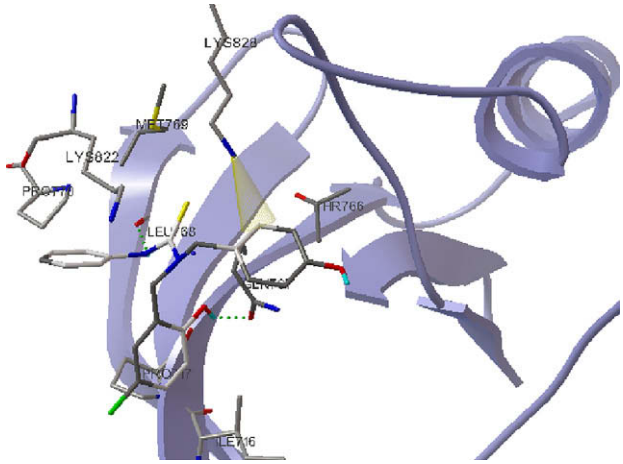


Figure 1. Molecular docking modeling of compounds **7b** with EGFR kinase: The N–H groups of **7b** project toward the side chain carbonyl group of Leu 768, the hydroxyl group of **7b** also forms hydrogen bond with amino hydrogen of Gln767, and there is a π-cation interaction between R^3-benzyl ring of compound **7b** and Lys828.

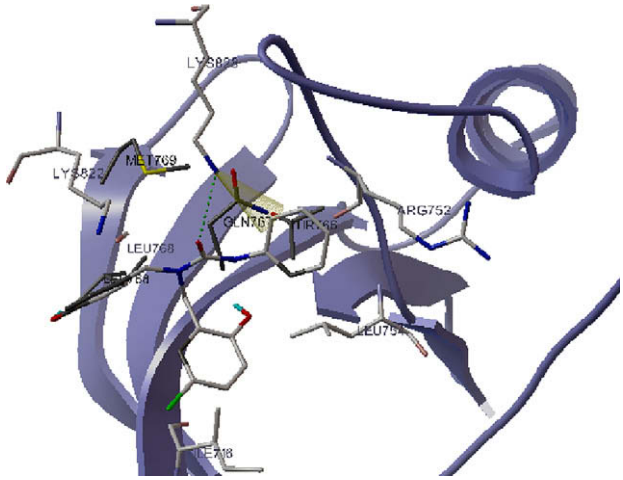


Figure 2. Molecular docking modeling of compounds **7a** with EGFR kinase.

important role in stabilizing the three dimensional structure of a protein.[23]

The phenyl group in **7b** projects into a hydrophobic region, which is comprised of the side chains of Pro 770, Lys 822 and Met 769, that was important for the potent inhibitory activity of **7b**, the decrease of activity caused by the replacement of the terminal aryl group with alkyl group (**13a–18a** and **13b–18b**) is probably due to the effect of an inferior interaction of the alkyl groups with the hydrophobic region. Also, in these thiourea compounds, replacement of a methyl group or a bromide at R^2 position by a chloride group resulted in the decrease of EGFR inhibitory activity. This suggested that compounds with strong electron-drawing groups on aromatic ring may benefit for the molecular binding between the compounds with EGFR.

The in vitro antiproliferative activity of the synthesized *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas was studied on a panel of one human tumor cell line (MCF-7), which overexpresses EGFR and, to a less extent HER-2. The compounds which have potent inhibitory activity of EGFR and HER-2 displayed high antiproliferative activity against MCF-7, indicating that these series ureas and thioureas were potent inhibitor of EGFR and HER-2 as antitumor agents. In particular, compounds **7b** has demonstrated significant efficacy in tumor growth inhibition and displayed favorable EGFR and HER-2 inhibitory activity.

3. Conclusion

Two series of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas and thioureas that may function as inhibitors of EGFR and HER-2 kinases have been prepared, a number of synthesized compounds displayed potent EGFR and HER-2 inhibitory. Extensive SAR studies led to the conclusion that compounds containing a R^1 and/or R^2 = Br or Cl are consistently more potent than those with a methyl group, the alkyl chain group in the urea and thioureas led to decrease in EGFR and HER-2 inhibitory activity. The EGFR molecular docking model suggested that the urea N–H groups of thiourea project toward carbonyl group of Leu 768, π-cation interaction between R^3-benzyl ring and Lys828 may an important role in stabilizing the three dimensional structure of a protein. The decrease of activity caused by the replacement of the terminal aryl group with alkyl group (**13a–18a** and **13b–18b**) is probably due to the effect of an inferior interaction of the alkyl groups with the hydrophobic region. Antiproliferative assay results indicating that these series of urea and thioureas own high inhibitory activity against MCF-7, in particular, thiourea **7b** has demonstrated significant EGFR inhibitory activity and antiproliferative activity as a potential anticancer agent.

4. Experimental

4.1. Chemistry general

All the NMR spectra were recorded on a Bruker DRX 500 or DPX 300 model Spectrometer in DMSO-d_6. Chemical shifts (δ) for ^{1}H NMR spectra were reported in parts per million to residual solvent protons. Melting points were measured on a Boetius micro melting point apparatus. The ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. All chemicals and reagents used in current study were of analytical grade. TLC was run on the silica gel coated aluminum sheets (Silica Gel 60 $GF_{254,}$ E. Merk, Germany) and visualized in UV light (254 nm).

4.2. General procedure for amines preparations (1–18)

To a solution of R^1 and R^2 substituted salicylaldehyde (2 mmol) and the primary substituted amines (2 mmol) were added, in ethanol. The mixture was refluxed, with stirring and the water was collected in a Dean–Stark apparatus. The reaction was monitored by TLC. The products were filtered and recrystallized in ethanol. Without further purification, to an ethanolic solution of synthesized Schiff bases (2 mmol), $NaBH_4$ (2 mmol) was slowly added in an ice bath with stirring. The mixture was refluxed for 3–6 h, then the solvent was evaporated and water (2 mL) was added. The product extraction was carried out with $CHCl_3$ (3 × 2 mL). The organic layer was dried over Na_2SO_4, and after solvent evaporation the residue was purified by flash column chromatography (Silica Gel 60, 35–70 mesh) using $CHCl_3$ as the eluent.

4.2.1. 4-Chloro-2-((4-fluorobenzylamino)methyl)phenol (1)

Yellow oil. Yield: 84%; ^{1}H NMR (DMSO-d_6) δ = 1.58 (s, 1H), 3.64 (s, 2H), 3.83 (s, 2H), 6.82 (d, J = 8.4 Hz, 1H), 7.20–7.32 (m, 6H), 13.72 (s, 1H). MS (ESI) $C_{14}H_{14}ClFNO$ $[M+H]^+$ 266.1. Anal. Calcd for $C_{14}H_{13}ClFNO$: C, 63.28; H, 4.93; Cl, 13.34; F, 7.15; N, 5.27. Found: C, 63.25; H, 4.92; Cl, 13.31; F, 7.14; N, 5.26.

4.2.2. 4-Bromo-2-((4-fluorobenzylamino)methyl)phenol (2)

Yellow oil. Yield: 81%; ^{1}H NMR (DMSO-d_6) δ = 1.55 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 6.77 (d, J = 8.4 Hz, 1H), 7.15–7.28 (m, 6H),

13.76 (s, 1H). MS (ESI) $C_{14}H_{14}BrFNO$ $[M+H]^+$ 310.0. Anal. Calcd for $C_{14}H_{13}BrFNO$: C, 54.21; H, 4.22; Br, 25.76; F, 6.13; N, 4.52. Found: C, 54.18; H, 4.21; Br, 25.72; F, 6.11; N, 4.49.

4.2.3. 2-((4-Fluorobenzylamino)methyl)-4-methylphenol (3)

Yellow oil. Yield: 93%; ^1H NMR (DMSO-d_6) δ = 1.58 (s, 1H), 2.48 (s, 3H), 3.65 (s, 2H), 3.86 (s, 2H), 6.85 (s, 1H), 7.21–7.34 (m, 6H), 13.73 (s, 1H). MS (ESI) $C_{15}H_{17}FNO$ $[M+H]^+$ 246.1. Anal. Calcd for $C_{15}H_{16}FNO$: C, 73.45; H, 6.57; F, 7.75; N, 5.71. Found: C, 73.41; H, 6.52; F, 7.73; N, 5.74.

4.2.4. 2,4-Dichloro-6-((4-fluorobenzylamino)methyl)phenol (4)

Yellow oil. Yield: 74%; ^1H NMR (DMSO-d_6) δ = 1.56 (s, 1H), 3.66 (s, 2H), 3.85 (s, 2H), 7.18–7.29 (m, 6H). MS (ESI) $C_{14}H_{13}Cl_2FNO$ $[M+H]^+$ 300.0. Anal. Calcd for $C_{14}H_{12}Cl_2FNO$: C, 56.02; H, 4.03; Cl, 23.62; F, 6.33; N, 4.67. Found: C, 55.94; H, 4.05; Cl, 23.63; F, 6.31; N, 4.65.

4.2.5. 2,4-Dibromo-6-((4-fluorobenzylamino)methyl)phenol (5)

Yellow oil. Yield: 78%; ^1H NMR (DMSO-d_6) δ = 1.55 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 7.15–7.28 (m, 6H). MS (ESI) $C_{14}H_{13}Br_2FNO$ $[M+H]^+$ 387.9. Anal. Calcd for $C_{14}H_{12}Br_2FNO$: C, 43.22; H, 3.11; Br, 41.08; F, 4.88; N, 3.60. Found: C, 43.26; H, 3.07; Br, 41.03; F, 4.84; N, 3.61.

4.2.6. 2-((4-Fluorobenzylamino)methyl)-4,6-dimethylphenol (6)

Yellow oil. Yield: 83%; ^1H NMR (DMSO-d_6) δ = 1.58 (s, 1H), 2.26 (s, 3H), 2.48 (s, 3H), 3.65 (s, 2H), 3.86 (s, 2H), 7.21–7.34 (m, 6H). MS (ESI) $C_{16}H_{19}FNO$ $[M+H]^+$ 260.1. Anal. Calcd for $C_{16}H_{18}FNO$: C, 74.11; H, 7.00; F, 7.33; N, 5.40. Found: C, 74.09; H, 7.03; F, 7.31; N, 5.46.

4.2.7. 4-Chloro-2-((4-hydroxybenzylamino)methyl)phenol (7)

Yellow oil. Yield: 90%; ^1H NMR (DMSO-d_6) δ = 1.56 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 6.83 (s, 1H), 7.09–7.22 (m, 4H), 7.27–7.30 (m, 2H), 13.73 (s, 1H). MS (ESI) $C_{14}H_{15}ClNO_2$ $[M+H]^+$ 264.1. Anal. Calcd for $C_{14}H_{14}ClNO_2$: C, 63.76; H, 5.35; Cl, 13.44; N, 5.31. Found: C, 63.72; H, 5.31; Cl, 13.42; N, 5.33.

4.2.8. 4-Bromo-2-((4-hydroxybenzylamino)methyl)phenol (8)

Yellow oil. Yield: 71%; ^1H NMR (DMSO-d_6) δ = 1.54 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 6.81 (s, 1H), 7.07–7.21 (m, 4H), 7.25–7.28 (m, 2H), 13.66 (s, 1H). MS (ESI) $C_{14}H_{15}BrNO_2$ $[M+H]^+$ 308.0. Anal. Calcd for $C_{14}H_{14}BrNO_2$: C, 54.56; H, 4.58; Br, 25.93; N, 4.55. Found: C, 54.52; H, 4.51; Br, 25.92; N, 4.56.

4.2.9. 2-((4-Hydroxybenzylamino)methyl)-4-methylphenol (9)

Yellow oil. Yield: 82%; ^1H NMR (DMSO-d_6) δ = 1.56 (s, 1H), 2.48 (s, 3H), 3.65 (s, 2H), 3.81 (s, 2H), 6.85 (s, 1H), 7.10–7.23 (m, 4H), 7.29–7.32 (m, 2H), 13.71 (s, 1H). MS (ESI) $C_{15}H_{18}NO_2$ $[M+H]^+$ 244.1. Anal. Calcd for $C_{15}H_{17}NO_2$: C, 74.05; H, 7.04; N, 5.76. Found: C, 74.01; H, 7.03; N, 5.72.

4.2.10. 2,4-Dichloro-6-((4-hydroxybenzylamino)methyl)phenol (10)

Yellow oil. Yield: 78%; ^1H NMR (DMSO-d_6) δ = 1.55 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 7.09–7.22 (m, 4H), 7.27–7.30 (m, 2H), 13.73 (s, 1H). MS (ESI) $C_{14}H_{14}Cl_2NO_2$ $[M+H]^+$ 298.0. Anal. Calcd for $C_{14}H_{13}Cl_2NO_2$: C, 56.39; H, 4.39; Cl, 23.78; N, 4.70. Found: C, 56.33; H, 4.35; Cl, 23.76; N, 4.67.

4.2.11. 2,4-Dibromo-6-((4-hydroxybenzylamino)methyl)phenol (11)

Yellow oil. Yield: 79%; ^1H NMR (DMSO-d_6) δ = 1.56 (s, 1H), 3.63 (s, 2H), 3.81 (s, 2H), 7.07–7.21 (m, 4H), 7.25–7.28(m, 2H), 13.66 (s, 1H). MS (ESI) $C_{14}H_{14}Br_2NO_2$ $[M+H]^+$ 385.9. Anal. Calcd for $C_{14}H_{13}Br_2NO_2$: C, 43.44; H, 3.39; Br, 41.29; N, 3.62. Found: C, 43.41; H, 3.32; Br, 41.24; N, 3.60.

4.2.12. 2-((4-Hydroxybenzylamino)methyl)-4,6-dimethylphenol (12)

Yellow oil. Yield: 84%; ^1H NMR (DMSO-d_6) δ = 1.56 (s, 1H), 2.31 (s, 3H), 2.47 (s, 3H), 3.65 (s, 2H), 3.81 (s, 2H), 7.10–7.23 (m, 4H), 7.29–7.32 (m, 2H), 13.71 (s, 1H). MS (ESI) $C_{16}H_{20}NO_2$ $[M+H]^+$ 258.1. Anal. Calcd for $C_{16}H_{19}NO_2$: C, 74.68; H, 7.44; N, 5.44. Found: C, 74.65; H, 7.41; N, 5.42.

4.2.13. 2-((Butylamino)methyl)-4-chlorophenol (13)

Yellow oil. Yield: 70%; ^1H NMR (DMSO-d_6) δ = 0.91 (t, J = 7.5 Hz, 3H), 1.29–1.41 (m, 2H), 1.52 (s, 1H), 1.57–1.66 (m, 2H), 3.30–3.32 (m, 2H), 3.63 (s, 2H), 6.87 (d, J = 9 Hz, 1H), 7.31–7.35 (m, 1H), 7.54(t, J = 4.5 Hz, 1H), 13.76 (s, 1H). MS (ESI) $C_{11}H_{17}ClNO$ $[M+H]^+$ 214.1. Anal. Calcd for $C_{11}H_{16}ClNO$: C, 61.82; H, 7.55; Cl, 16.59; N, 6.55. Found: C, 61.81; H, 7.52; Cl, 16.56; N, 6.58.

4.2.14. 4-Bromo-2-((butylamino)methyl)phenol (14)

Yellow oil. Yield: 76%; ^1H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.29–1.41 (m, 2H), 1.52 (s, 1H), 1.57–1.66 (m, 2H), 3.30–3.33 (m, 2H), 3.63 (s, 2H), 6.85 (d, J = 9 Hz, 1H), 7.29–7.33 (m, 1H), 7.53 (t, J = 4.5 Hz, 1H), 13.71 (s, 1H). MS (ESI) $C_{11}H_{17}BrNO$ $[M+H]^+$ 258.0. Anal. Calcd for $C_{11}H_{16}BrNO$: C, 51.18; H, 6.25; Br, 30.95; N, 5.43. Found: C, 51.13; H, 6.22; Br, 30.91; N, 5.45.

4.2.15. 2-((Butylamino)methyl)-4-methylphenol (15)

Yellow oil. Yield: 67%; ^1H NMR (DMSO-d_6) δ = 0.91 (t, J = 7.5 Hz, 3H), 1.28–1.39 (m, 2H), 1.51 (s, 1H), 1.57–1.66 (m, 2H), 2.44 (s, 3H), 3.32–3.35 (s, 2H), 3.63 (s, 2H), 6.90 (d, J = 9 Hz, 1H), 7.33–7.38 (m, 1H), 7.57 (t, J = 4.5 Hz, 1H), 13.77 (s, 1H). MS (ESI) $C_{12}H_{20}NO$ $[M+H]^+$ 194.2. Anal. Calcd for $C_{12}H_{19}NO$: C, 74.57; H, 9.91; N, 7.25. Found: C, 74.51; H, 9.92; N, 7.23.

4.2.16. 2-((Butylamino)methyl)-4,6-dichlorophenol (16)

Yellow oil. Yield: 72%; ^1H NMR (DMSO-d_6) δ = 0.91 (t, J = 7.5 Hz, 3H), 1.29–1.41 (m, 2H), 1.52 (s, 1H), 1.57–1.66 (m, 2H), 3.30–3.34 (m, 2H), 3.63 (s, 2H), 7.32 (s, 1H), 7.52 (t, J = 4.5 Hz, 1H), 13.76 (s, 1H). MS (ESI) $C_{11}H_{16}Cl_2NO$ $[M+H]^+$ 248.1. Anal. Calcd for $C_{11}H_{15}Cl_2NO$: C, 53.24; H, 6.09; Cl, 28.57; N, 5.64. Found: C, 53.20; H, 6.05; Cl, 28.56; N, 5.65.

4.2.17. 2,4-Dibromo-6-((butylamino)methyl)phenol (17)

Yellow oil. Yield: 60%; ^1H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.29–1.41 (m, 2H), 1.52 (s, 1H), 1.57–1.66 (m, 2H), 3.28–3.31 (m, 2H), 3.63 (s, 2H), 7.30 (s, 1H), 7.53 (t, J = 4.5 Hz, 1H), 13.71 (s, 1H). MS (ESI) $C_{11}H_{16}Br_2NO$ $[M+H]^+$ 336.0. Anal. Calcd for $C_{11}H_{15}Br_2NO$: C, 39.20; H, 4.49; Br, 47.41; N, 4.16. Found: C, 39.17; H, 4.45; Br, 47.37; N, 4.13.

4.2.18. 2-((Butylamino)methyl)-4,6-dimethylphenol (18)

Yellow oil. Yield: 71%; ^1H NMR (DMSO-d_6) δ = 0.91 (t, J = 7.5 Hz, 3H), 1.28–1.39 (m, 2H), 1.51 (s, 1H), 1.57–1.66 (m, 2H), 2.24 (s, 3H), 2.44 (s, 3H), 3.28–3.31 (m, 2H), 3.63 (s, 2H), 7.37 (s, 1H), 7.57 (t, J = 4.5 Hz, 1H), 13.77 (s, 1H). MS (ESI) $C_{13}H_{22}NO$ $[M+H]^+$ 208.2. Anal. Calcd for $C_{13}H_{21}NO$: C, 75.32; H, 10.21; N, 6.76. Found: C, 75.26; H, 10.27; N, 6.71.

4.3. General procedure for ureas (1a–18a) and thioureas (1b–18b) preparations

To chloroform solution of amines (**1–18**) (1.5 mmol), phenylisocyanate or phenylisothiocyanate (1.5 mmol) was slowly added with stirring. The mixture was refluxed for 2–4 h. After this time, the product was filtered and purified by recrystallization from ethanol.

4.3.1. 1-(5-Chloro-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylurea (1a)

Mp 172–173 °C; yield: 85%; ^{1}H NMR (DMSO-d_6) δ = 4.39 (s, 2H), 4.72 (s, 2H), 6.81 (d, J = 8.4 Hz, 1H), 7.03 (s, 1H), 7.08–7.18 (m, 4H), 7.24–7.34 (m, 6H), 9.61 (s, 1H), 10.11 (s, 1H). MS (ESI) $C_{21}H_{19}ClFN_2O_2$ $[M+H]^+$ 385.1. Anal. Calcd for $C_{21}H_{18}ClFN_2O_2$: C, 65.54; H, 4.71; Cl, 9.21; F, 4.94; N, 7.28. Found: C, 65.48; H, 4.67; Cl, 9.17; F, 4.92; N, 7.29.

4.3.2. 1-(5-Bromo-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylurea (2a)

Mp 158–159 °C; yield: 92%; ^{1}H NMR (DMSO-d_6) δ = 4.42 (s, 2H), 4.73 (s, 2H), 6.80 (d, J = 8.4 Hz, 1H), 7.07–7.43 (m, 11H), 9.44 (s, 1H), 10.12 (s, 1H). MS (ESI) $C_{21}H_{19}BrFN_2O_2$ $[M+H]^+$ 429.1. Anal. Calcd for $C_{21}H_{18}BrFN_2O_2$: C, 58.75; H, 4.23; Br, 18.61; F, 4.43; N, 6.53. Found: C, 58.70; H, 4.22; Br, 18.56; F, 4.39; N, 6.52.

4.3.3. 1-(4-Fluorobenzyl)-1-(2-hydroxy-5-methylbenzyl)-3-phenylurea (3a)

Mp 172–173 °C; yield: 86%; ^{1}H NMR (DMSO-d_6) δ = 2.46 (s, 3H), 4.43 (s, 2H), 4.70 (s, 2H), 6.83 (d, J = 8.4 Hz, 1H), 7.01 (s, 1H), 6.98–7.12 (m, 2H), 7.23–7.38 (m, 8H), 9.42 (s, 1H), 10.08 (s, 1H). MS (ESI) $C_{22}H_{22}FN_2O_2$ $[M+H]^+$ 365.2. Anal. Calcd for $C_{22}H_{21}FN_2O_2$: C, 72.51; H, 5.81; F, 5.21; N, 7.69. Found: C, 72.48; H, 5.83; F, 5.16; N, 7.63.

4.3.4. 1-(3,5-Dichloro-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylurea (4a)

Mp 138–139 °C; yield: 95%; ^{1}H NMR (DMSO-d_6) δ = 4.29 (s, 2H), 4.76 (s, 2H), 6.90–7.23 (m, 11H), 7.55 (s, 1H). MS (ESI) $C_{21}H_{18}Cl_2FN_2O_2$ $[M+H]^+$ 419.1. Anal. Calcd for $C_{21}H_{17}Cl_2FN_2O_2$: C, 60.16; H, 4.09; Cl, 16.91; F, 4.53; N, 6.68. Found: C, 60.12; H, 4.05; Cl, 16.89; F, 4.51; N, 6.65.

4.3.5. 1-(3,5-Dibromo-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylurea (5a)

Mp 133–134 °C; yield: 80%; ^{1}H NMR (DMSO-d_6) δ = 4.26 (s, 2H), 4.80 (s, 2H), 6.93–7.24 (m, 11H), 7.55 (s, 1H). MS (ESI) $C_{21}H_{18}Br_2FN_2O_2$ $[M+H]^+$ 507.0. Anal. Calcd for $C_{21}H_{17}Br_2FN_2O_2$: C, 49.63; H, 3.37; Br, 31.45; F, 3.74; N, 5.51. Found: C, 49.59; H, 3.35; Br, 31.43; F, 3.71; N, 5.53.

4.3.6. 1-(4-Fluorobenzyl)-1-(2-hydroxy-3,5-dimethylbenzyl)-3-phenylthiourea (6a)

Mp 128–129 °C; yield: 77%; ^{1}H NMR (DMSO-d_6) δ = 2.23 (s, 3H), 2.45 (s, 3H), 4.28 (s, 2H), 4.72 (s, 2H), 6.96–7.29 (m, 11H), 7.55 (s, 1H). MS (ESI) $C_{23}H_{24}FN_2O_2$ $[M+H]^+$ 379.2. Anal. Calcd for $C_{23}H_{23}FN_2O_2$: C, 73.00; H, 6.13; F, 5.02; N, 7.40. Found: C, 72.95; H, 6.11; F, 5.03; N, 7.36.

4.3.7. 1-(5-Chloro-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (7a)

Mp 164–165 °C; yield: 87%; ^{1}H NMR (DMSO-d_6) δ = 4.42 (s, 2H), 4.68 (s, 2H), 6.81 (d, J = 8.4 Hz, 1H), 6.98 (s, 1H), 7.08–7.31 (m, 10H), 9.48 (s, 1H), 10.01 (s, 1H). MS (ESI) $C_{21}H_{20}ClN_2O_3$ $[M+H]^+$ 383.1. Anal. Calcd for $C_{21}H_{19}ClN_2O_3$: C, 65.88; H, 5.00; Cl, 9.26; N, 7.32. Found: C, 65.83; H, 4.95; Cl, 9.22; N, 7.29.

4.3.8. 1-(5-Bromo-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (8a)

Mp 155–156 °C; yield: 85%; ^{1}H NMR (DMSO-d_6) δ = 4.46 (s, 2H), 4.85 (s, 2H), 6.80 (d, J = 8.4 Hz, 1H), 7.05–7.40 (m, 11H), 9.48 (s, 1H), 10.11 (s, 1H). MS (ESI) $C_{21}H_{20}BrN_2O_3$ $[M+H]^+$ 427.1. Anal. Calcd for $C_{21}H_{19}BrN_2O_3$: C, 59.03; H, 4.48; Br, 18.70; N, 6.56. Found: C, 59.01; H, 4.42; Br, 18.66; N, 6.53.

4.3.9. 1-(2-Hydroxy-5-methylbenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (9a)

Mp 148–149 °C; yield: 77%; ^{1}H NMR (DMSO-d_6) δ = 2.49 (s, 3H), 4.43 (s, 2H), 4.76 (s, 2H), 6.83 (d, J = 8.4 Hz, 1H), 7.01 (s, 1H), 7.05–7.13 (m, 3H), 7.24–7.38 (m, 7H), 9.43 (s, 1H), 10.07 (s, 1H). MS (ESI) $C_{22}H_{23}N_2O_3$ $[M+H]^+$ 363.2. Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.89; H, 6.09; N, 7.71.

4.3.10. 1-(3,5-Dichloro-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (10a)

Mp 141–142 °C; yield: 72%; ^{1}H NMR (DMSO-d_6) δ = 4.27 (s, 2H), 4.68 (s, 2H), 6.88–7.21 (m, 10H), 7.55 (s, 1H), 10.41 (s, 1H). MS (ESI) $C_{21}H_{19}Cl_2N_2O_3$ $[M+H]^+$ 417.1. Anal. Calcd for $C_{21}H_{18}Cl_2N_2O_3$: C, 60.44; H, 4.35; Cl, 16.99; N, 6.71. Found: C, 60.42; H, 4.31; Cl, 16.95; N, 6.67.

4.3.11. 1-(3,5-Dibromo-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (11a)

Mp 130–131 °C; yield: 79%; ^{1}H NMR (DMSO-d_6) δ = 4.39 (s, 2H), 4.77 (s, 2H), 6.92–7.24 (m, 10H), 7.57 (s, 1H), 10.34 (s, 1H). MS (ESI) $C_{21}H_{19}Br_2N_2O_3$ $[M+H]^+$ 505.0. Anal. Calcd for $C_{21}H_{18}Br_2N_2O_3$: C, 49.83; H, 3.58; Br, 31.57; N, 5.53. Found: C, 49.85; H, 3.53; Br, 31.54; N, 5.51.

4.3.12. 1-(2-Hydroxy-3,5-dimethylbenzyl)-1-(4-hydroxybenzyl)-3-phenylurea (12a)

Mp 127–128 °C; yield: 81%; ^{1}H NMR (DMSO-d_6) δ = 2.32 (s, 3H), 2.50 (s, 3H), 4.38 (s, 2H), 4.73 (s, 2H), 7.00–7.32 (m, 10H), 7.57 (s, 1H), 10.37 (s, 1H). MS (ESI) $C_{23}H_{25}N_2O_3$ $[M+H]^+$ 377.1. Anal. Calcd for $C_{23}H_{24}N_2O_3$: C, 73.38; H, 6.43; N, 7.44. Found: C, 73.51; H, 6.25; N, 7.53.

4.3.13. 1-Butyl-1-(5-chloro-2-hydroxybenzyl)-3-phenylurea (13a)

Mp 123–124 °C; yield: 67%; ^{1}H NMR (DMSO-d_6) δ = 0.87 (t, J = 7.5 Hz, 3H), 1.12–1.18 (m, 2H), 1.52–1.62 (m, 2H), 3.04–3.09 (m, 1H), 3.28 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 6.84 (d, J = 9 Hz, 1H), 7.08–7.14 (m, 3H), 7.23–7.30 (m, 4H), 9.17 (s, 1H), 10.06 (s, 1H). MS (ESI) $C_{18}H_{22}ClN_2O_2$ $[M+H]^+$ 333.1. Anal. Calcd for $C_{18}H_{21}ClN_2O_2$: C, 64.96; H, 6.36; Cl, 10.65; N, 8.42. Found: C, 64.93; H, 6.32; Cl, 10.61; N, 8.40.

4.3.14. 1-(5-Bromo-2-hydroxybenzyl)-1-butyl-3-phenylurea (14a)

Mp 121–122 °C; yield: 71%; ^{1}H NMR (DMSO-d_6) δ = 0.89 (t, J = 7.5 Hz, 3H), 1.04–1.12 (m, 2H), 1.54–1.64 (m, 2H), 3.12–3.16 (m, 1H), 3.23 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 6.84 (d, J = 9 Hz, 1H), 7.08–7.14 (m, 3H), 7.21–7.29 (m, 4H), 9.18 (s, 1H), 10.04 (s, 1H). MS (ESI) $C_{18}H_{22}BrN_2O_2$ $[M+H]^+$ 377.1. Anal. Calcd for $C_{18}H_{21}BrN_2O_2$: C, 57.30; H, 5.61; Br, 21.18; N, 7.43. Found: C, 57.28; H, 5.57; Br, 21.17; N, 7.41.

4.3.15. 1-Butyl-1-(2-hydroxy-5-methylbenzyl)-3-phenylurea (15a)

Mp 127–128 °C; yield: 68%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.05–1.11 (m, 2H), 1.53–1.62 (m, 2H), 2.45 (s, 3H), 3.03–3.08 (m, 1H), 3.27 (t, J = 7.5 Hz, 2H), 4.96 (s, 2H), 6.83 (d, J = 9 Hz, 1H), 7.10–7.17 (m, 3H), 7.23–7.34 (m, 4H), 9.21 (s, 1H), 10.07 (s, 1H). MS (ESI) $C_{19}H_{25}N_2O_2$ $[M+H]^+$ 313.2. Anal. Calcd for $C_{19}H_{24}N_2O_2$: C, 73.05; H, 7.74; N, 8.97. Found: C, 73.08; H, 7.67; N, 8.93.

4.3.16. 1-Butyl-1-(3,5-dichloro-2-hydroxybenzyl)-3-phenylturea (16a)

Mp 136–137 °C; yield: 61%; ^{1}H NMR (DMSO-d_6) δ = 0.88 (t, J = 7.5 Hz, 3H), 1.02–1.10 (m, 2H), 1.51–1.61 (m, 2H), 3.01–3.05

(m, 1H), 3.20 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 7.04–7.11 (m, 3H), 7.23–7.30 (m, 4H), 7.55 (s, 1H). MS (ESI) $C_{18}H_{21}Cl_2N_2O_2$ $[M+H]^+$ 367.1. Anal. Calcd for $C_{18}H_{20}Cl_2N_2O_2$: C, 58.86; H, 5.49; Cl, 19.31; N, 7.63. Found: C, 58.86; H, 5.46; Cl, 19.29; N, 7.61.

4.3.17. 1-Butyl-1-(3,5-dibromo-2-hydroxybenzyl)-3-phenylurea (17a)

Mp 129–130 °C; yield: 55%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.04–1.12 (m, 2H), 1.53–1.59 (m, 2H), 3.03–3.06 (m, 1H), 3.24 (t, J = 7.5 Hz, 2H), 4.96 (s, 2H), 7.08–7.14 (m, 3H), 7.22–7.33(m, 4H), 7.55 (s, 1H). MS (ESI) $C_{18}H_{21}Br_2N_2O_2$ $[M+H]^+$ 455.0. Anal. Calcd for $C_{18}H_{20}Br_2N_2O_2$: C, 47.39; H, 4.42; Br, 35.03; N, 6.14. Found: C, 47.36; H, 4.41; Br, 35.99; N, 6.10.

4.3.18. 1-Butyl-1-(2-hydroxy-3,5-dimethylbenzyl)-3-phenylurea (18a)

Mp 133–134 °C; yield: 59%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.02–1.09 (m, 2H), 1.50–1.58 (m, 2H), 2.32 (s, 3H), 2.50 (s, 3H), 3.04–3.08 (m, 1H), 3.23 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 7.10–7.15 (m, 3H), 7.24–7.35 (m, 4H), 7.55 (s, 1H). MS (ESI) $C_{20}H_{27}N_2O_2$ $[M+H]^+$ 327.2. Anal. Calcd for $C_{20}H_{26}N_2O_2$: C, 73.59; H, 8.03; N, 8.58. Found: C, 73.58; H, 8.00; N, 8.61.

4.3.19. 1-(5-Chloro-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylthiourea (1b)

Mp 185–186 °C; yield: 82%; ^{1}H NMR (DMSO-d_6) δ = 4.88 (s, 2H), 5.11 (s, 2H), 6.81 (d, J = 8.4 Hz, 1H), 7.04 (s, 1H), 7.12–7.21 (m, 4H), 7.29–7.37 (m, 6H), 9.63 (s, 1H), 10.14 (s, 1H). MS (ESI) $C_{21}H_{19}ClFN_2OS$ $[M+H]^+$ 401.1. Anal. Calcd for $C_{21}H_{18}ClFN_2OS$: C, 62.92; H, 4.53; Cl, 8.84; F, 4.74; N, 6.99; S, 8.00. Found: C, 62.91; H, 4.55; Cl, 8.78; F, 4.71; N, 6.96; S, 8.02.

4.3.20. 1-(5-Bromo-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylthiourea (2b)

Mp 170–171 °C; yield: 85%; ^{1}H NMR (DMSO-d_6) δ = 4.88 (s, 2H), 5.19 (s, 2H), 6.80 (d, J = 8.4 Hz, 1H), 7.11–7.46 (m, 11H), 9.46 (s, 1H), 10.13 (s, 1H). MS (ESI) $C_{21}H_{19}BrFN_2OS$ $[M+H]^+$ 445.0. Anal. Calcd for $C_{21}H_{18}BrFN_2OS$: C, 56.64; H, 4.07; Br, 17.94; F, 4.27; N, 6.29; S, 7.20. Found: C, 56.60; H, 4.09; Br, 17.97; F, 4.24; N, 6.23; S, 7.16.

4.3.21. 1-(4-Fluorobenzyl)-1-(2-hydroxy-5-methylbenzyl)-3-phenylthiourea (3b)

Mp 176–177 °C; yield: 88%; ^{1}H NMR (DMSO-d_6) δ = 2.46 (s, 3H), 4.90 (s, 2H), 5.10 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 7.04 (s, 1H), 7.08–7.16 (m, 2H), 7.29–7.43 (m, 8H), 9.47 (s, 1H), 10.08 (s, 1H). MS (ESI) $C_{22}H_{22}FN_2OS$ $[M+H]^+$ 381.1. Anal. Calcd for $C_{22}H_{21}FN_2OS$: C, 69.45; H, 5.56; F, 4.99; N, 7.36; S, 8.43. Found: C, 69.48; H, 5.52; F, 4.95; N, 7.33; S, 8.40.

4.3.22. 1-(3,5-Dichloro-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylthiourea (4b)

Mp 157–159 °C; yield: 78%; ^{1}H NMR (DMSO-d_6) δ = 4.78 (s, 2H), 5.17 (s, 2H), 6.92–7.25 (m, 11H), 7.56 (s, 1H). MS (ESI) $C_{21}H_{18}Cl_2FN_2OS$ $[M+H]^+$ 435.0. Anal. Calcd for $C_{21}H_{17}Cl_2FN_2OS$: C, 57.94; H, 3.94; Cl, 16.29; F, 4.36; N, 6.43; S, 7.37. Found: C, 57.96; H, 3.91; Cl, 16.27; F, 4.34; N, 6.41; S, 7.35.

4.3.23. 1-(3,5-Dibromo-2-hydroxybenzyl)-1-(4-fluorobenzyl)-3-phenylthiourea (5b)

Mp 144–145 °C; yield: 74%; ^{1}H NMR (DMSO-d_6) δ = 4.75 (s, 2H), 5.19 (s, 2H), 6.97–7.28 (m, 11H), 7.57 (s, 1H). MS (ESI) $C_{21}H_{18}Br_2FN_2OS$ $[M+H]^+$ 523.9. Anal. Calcd for $C_{21}H_{17}Br_2FN_2OS$: C, 48.11; H, 3.27; Br, 30.48; F, 3.62; N, 5.34; S, 6.12. Found: C, 48.14; H, 3.28; Br, 30.44; F, 3.60; N, 5.32; S, 6.11.

4.3.24. 1-(4-Fluorobenzyl)-1-(2-hydroxy-3,5-dimethylbenzyl)-3-phenylthiourea (6b)

Mp 136–137 °C; yield: 72%; ^{1}H NMR (DMSO-d_6) δ = 2.24 (s, 3H), 2.47 (s, 3H), 4.77 (s, 2H), 5.13 (s, 2H), 7.04–7.35 (m, 11H), 7.57 (s, 1H). MS (ESI) $C_{23}H_{24}FN_2OS$ $[M+H]^+$ 395.2. Anal. Calcd for $C_{23}H_{23}FN_2OS$: C, 70.02; H, 5.88; F, 4.82; N, 7.10; S, 8.13. Found: C, 70.05; H, 5.81; F, 4.86; N, 7.11; S, 8.15.

4.3.25. 1-(5-Chloro-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (7b)

Mp 168–169 °C; yield: 81%; ^{1}H NMR (DMSO-d_6) δ = 4.88 (s, 2H), 5.11 (s, 2H), 6.81 (d, J = 8.4 Hz, 1H), 7.04 (s, 1H), 7.12–7.37 (m, 10H), 9.48 (s, 1H), 10.01 (s, 1H). MS (ESI) $C_{21}H_{20}ClN_2O_2S$ $[M+H]^+$ 399.1. Anal. Calcd for $C_{21}H_{19}ClN_2O_2S$: C, 63.23; H, 4.80; Cl, 8.89; N, 7.02; S, 8.04. Found: C, 63.21; H, 4.83; Cl, 8.82; N, 6.98; S, 8.03.

4.3.26. 1-(5-Bromo-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (8b)

Mp 161–162 °C; yield: 78%; ^{1}H NMR (DMSO-d_6) δ = 4.88 (s, 2H), 5.19 (s, 2H), 6.80 (d, J = 8.4 Hz, 1H), 7.11–7.46 (m, 11H), 9.48 (s, 1H), 10.11 (s, 1H). MS (ESI) $C_{21}H_{20}BrN_2O_2S$ $[M+H]^+$ 443.1. Anal. Calcd for $C_{21}H_{19}BrN_2O_2S$: C, 56.89; H, 4.32; Br, 18.02; N, 6.32; S, 7.23. Found: C, 56.87; H, 4.26; Br, 18.04; N, 6.30; S, 7.18.

4.3.27. 1-(2-Hydroxy-5-methylbenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (9b)

Mp 147–148 °C; yield: 83%; ^{1}H NMR (DMSO-d_6) δ = 2.46 (s, 3H), 4.90 (s, 2H), 5.10 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 7.04 (s, 1H), 7.08–7.16 (m, 3H), 7.29–7.43 (m, 7H), 9.44 (s, 1H), 10.07 (s, 1H). MS (ESI) $C_{22}H_{23}N_2O_2S$ $[M+H]^+$ 379.1. Anal. Calcd for $C_{22}H_{22}N_2O_2S$: C, 69.81; H, 5.86; N, 7.40; S, 8.47. Found: C, 69.80; H, 5.82; N, 7.42; S, 8.45.

4.3.28. 1-(3,5-Dichloro-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (10b)

Mp 152–153 °C; yield: 68%; ^{1}H NMR (DMSO-d_6) δ = 4.75 (s, 2H), 5.14 (s, 2H), 6.92–7.25 (m, 10H), 7.56 (s, 1H), 10.43 (s, 1H). MS (ESI) $C_{21}H_{19}Cl_2N_2O_2S$ $[M+H]^+$ 433.1. Anal. Calcd for $C_{21}H_{18}Cl_2N_2O_2S$: C, 58.20; H, 4.19; Cl, 16.36; N, 6.46; S, 7.40. Found: C, 58.22; H, 4.17; Cl, 16.33; N, 6.43; S, 7.38.

4.3.29. 1-(3,5-Dibromo-2-hydroxybenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (11b)

Mp 158–159 °C; yield: 65%; ^{1}H NMR (DMSO-d_6) δ = 4.78 (s, 2H), 5.16 (s, 2H), 6.97–7.28 (m, 10H), 7.57 (s, 1H), 10.41 (s, 1H). MS (ESI) $C_{21}H_{19}Br_2N_2O_2S$ $[M+H]^+$ 521.0. Anal. Calcd for $C_{21}H_{18}Br_2N_2O_2S$: C, 48.30; H, 3.47; Br, 30.60; N, 5.36; S, 6.14. Found: C, 48.28; H, 3.43; Br, 30.57; N, 5.34; S, 6.12.

4.3.30. 1-(2-Hydroxy-3,5-dimethylbenzyl)-1-(4-hydroxybenzyl)-3-phenylthiourea (12b)

Mp 145–146 °C; yield: 62%; ^{1}H NMR (DMSO-d_6) δ = 2.31 (s, 3H), 2.48 (s, 3H), 4.81 (s, 2H), 5.18 (s, 2H), 7.04–7.35 (m, 10H), 7.57 (s, 1H), 10.41 (s, 1H). MS (ESI) $C_{23}H_{25}N_2O_2S$ $[M+H]^+$ 393.2. Anal. Calcd for $C_{23}H_{24}N_2O_2S$: C, 70.38; H, 6.16; N, 7.14; S, 8.17. Found: C, 70.34; H, 6.15; N, 7.12; S, 8.14.

4.3.31. 1-Butyl-1-(5-chloro-2-hydroxybenzyl)-3-phenylthiourea (13b)

Mp 149–150 °C; yield: 56%; ^{1}H NMR (DMSO-d_6) δ = 0.88 (t, J = 7.5 Hz, 3H), 1.22–1.34 (m, 2H), 1.54–1.64 (m, 2H), 3.42–3.46 (m, 1H), 3.69 (t, J = 7.5 Hz, 2H), 4.96 (s, 2H), 6.84 (d, J = 9 Hz, 1H), 7.10–7.16 (m, 3H), 7.27–7.34 (m, 4H), 9.20 (s, 1H), 10.06 (s, 1H). MS (ESI) $C_{18}H_{22}ClN_2OS$ $[M+H]^+$ 349.1. Anal. Calcd for $C_{18}H_{21}ClN_2OS$: C, 61.97; H, 6.07; Cl, 10.16; N, 8.03; S, 9.19. Found: C, 61.96; H, 6.03; Cl, 10.14; N, 8.01; S, 9.15.

4.3.32. 1-(5-Bromo-2-hydroxybenzyl)-1-butyl-3-phenylthiourea (14b)

Mp 145–146 °C; yield: 55%; ^{1}H NMR (DMSO-d_6) δ = 0.89 (t, J = 7.5 Hz, 3H), 1.22–1.34 (m, 2H), 1.54–1.64 (m, 2H), 3.42–3.46 (m, 1H), 3.69 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 6.84 (d, J = 9 Hz, 1H), 7.08–7.14 (m, 3H), 7.25–7.33 (m, 4H), 9.18 (s, 1H), 10.04 (s, 1H). MS (ESI) $C_{18}H_{22}BrN_2OS$ $[M+H]^+$ 393.1. Anal. Calcd for $C_{18}H_{21}BrN_2OS$: C, 54.96; H, 5.38; Br, 20.31; N, 7.12; S, 8.15. Found: C, 54.94; H, 5.35; Br, 20.28; N, 7.10; S, 8.13.

4.3.33. 1-Butyl-1-(2-hydroxy-5-methylbenzyl)-3-phenylthiourea (15b)

Mp 142–142 °C; yield: 61%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.23–1.36 (m, 2H), 1.55–1.64 (m, 2H), 2.49 (s, 3H), 3.42–3.47 (m, 1H), 3.71 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 6.86 (d, J = 9 Hz, 1H), 7.12–7.19 (m, 3H), 7.28–7.39 (m, 4H), 9.21 (s, 1H), 10.07 (s, 1H). MS (ESI) $C_{19}H_{25}N_2OS$ $[M+H]^+$ 329.2. Anal. Calcd for $C_{19}H_{24}N_2OS$: C, 69.47; H, 7.36; N, 8.53; S, 9.76. Found: C, 69.41; H, 7.32; N, 8.56; S, 9.75.

4.3.34. 1-Butyl-1-(3,5-dichloro-2-hydroxybenzyl)-3-phenylthiourea (16b)

Mp 155–156 °C; yield: 51%; ^{1}H NMR (DMSO-d_6) δ = 0.88 (t, J = 7.5 Hz, 3H), 1.22–1.34 (m, 2H), 1.54–1.64 (m, 2H), 3.42–3.46 (m, 1H), 3.69 (t, J = 7.5 Hz, 2H), 4.96 (s, 2H), 7.10–7.16 (m, 3H), 7.27–7.34 (m, 4H), 7.55 (s, 1H). MS (ESI) $C_{18}H_{21}Cl_2N_2OS$ $[M+H]^+$ 383.1. Anal. Calcd for $C_{18}H_{20}Cl_2N_2OS$: C, 56.40; H, 5.26; Cl, 18.50; N, 7.31; S, 8.36. Found: C, 56.43; H, 5.23; Cl, 18.45; N, 7.28; S, 8.33%.

4.3.35. 1-Butyl-1-(3,5-dibromo-2-hydroxybenzyl)-3-phenylthiourea (17b)

Mp 148–149 °C;. yield: 47%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.23–1.36 (m, 2H), 1.55–1.64 (m, 2H), 3.42–3.47 (m, 1H), 3.71 (t, J = 7.5 Hz, 2H), 4.97 (s, 2H), 7.11–7.17 (m, 3H), 7.25–7.36(m, 4H), 7.55 (s, 1H). MS (ESI) $C_{18}H_{21}Br_2N_2OS$ $[M+H]^+$ 471.0. Anal. Calcd for $C_{18}H_{20}Br_2N_2OS$: C, 45.78; H, 4.27; Br, 33.84; N, 5.93; S, 6.79. Found: C, 45.74; H, 4.25; Br, 33.82; N, 5.91; S, 6.76.

4.3.36. 1-Butyl-1-(2-hydroxy-3,5-dimethylbenzyl)-3-phenylthiourea (18b)

Mp 136–137 °C; yield: 52%; ^{1}H NMR (DMSO-d_6) δ = 0.90 (t, J = 7.5 Hz, 3H), 1.23–1.36 (m, 2H), 1.55–1.64 (m, 2H), 2.32 (s, 3H), 2.49 (s, 3H), 3.42–3.47 (m, 1H), 3.71 (t, J = 7.5 Hz, 2H), 4.96 (s, 2H), 7.12–7.17 (m, 3H), 7.28–7.39 (m, 4H), 7.55 (s, 1H). MS (ESI) $C_{20}H_{27}N_2OS$ $[M+H]^+$ 343.2. Anal. Calcd for $C_{20}H_{26}N_2OS$: C, 70.14; H, 7.65; N, 8.18; S, 9.36. Found: C, 70.11; H, 7.62; N, 8.15; S, 9.33.

4.4. Preparation, and 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, 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 íM 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.[24]

Both EGFR and HER-2 kinase assays were set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Compounds **1a**–**18a** and **1b–18b** were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. In each well, 10 íL 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.

4.5. Cell proliferation assay

The antiproliferative activity was determined using a standard (MTT)-based colorimetric assay (Sigma), using 5-fluorouracil and genistein as reference drugs. 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 two independent experiments.

4.6. Molecular docking modeling

Molecular docking of compound **7b** 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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