Research Article

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Synthesis of novel thiourea-/urea-benzimidazole derivatives as anticancer agents

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Abstract: A new series of urea and thiourea derivatives containing benzimidazole group as potential anticancer agents have been designed and synthesized. The structures of the synthesized compounds were characterized and confirmed by spectroscopic techniques such as ^1H NMR, ^{13}C NMR, and mass spectrometry. *In vitro* anticancer assay against two breast cancer (BC) cell lines, MDA-MB-231^{ER(-)/PR(-)} and MCF-7^{ER(+)/PR(+)}, revealed that the cytotoxicity of 1-(2-(1*H*-benzo[d]imidazol-2-ylamino)ethyl)-3-*p*-tolylthiourea (**7b**) and 4-(1*H*-benzo[d]imidazol-2-yl)-*N*-(3-chlorophenyl)piperazine-1-carboxamide (**5d**) were higher in MCF-7 with IC₅₀ values of 25.8 and 48.3 μM , respectively, as compared with MDA-MB-231 cells. Furthermore, **7b** and **5d** were assessed for their apoptotic potential using 4',6-diamidino-2-phenylindole, acridine orange/ethidium bromide staining, and Caspase-3/7. After incubation with MCF-7, the compounds **7b** and **5d** induced apoptosis through caspase-3/7 activation. In conclusion, the compounds **7b** and **5d** are potential candidates for inducing apoptosis in different genotypic BC cell lines.

Keywords: benzimidazole, urea, thiourea, piperazine, anticancer activity, apoptosis

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1 Introduction

Benzimidazole is a very useful heterocycle for the development of molecules of pharmaceutical and biological interest. Compounds containing benzimidazole have been widely used in drug development and researchers around the world are actively seeking new uses and applications [1]. Benzimidazole derivatives have found applications in diverse therapeutical areas including antimicrobial [2–4], antifungal [5–8], antiviral [9,10], antitubercular [11–13], antidiabetic [14,15], anti-inflammatory [16,17], antihistaminic [18], antioxidant [19,20], and anticancer activities [21,22]. Moreover, benzimidazoles are one of the early classes of anticancer agents such as bendamustine Figure 1 [23]. In addition, many derivatives showed anticancer activity against human liver carcinoma (HEP-G2) cell lines [22], while others derivatives possess potent anticancer activity against other human cancer cell lines [16] such as MCF-7, THP-1, PC-3, and A-549. Thiourea and urea are classes of organic compounds which have a wide diversity and multiple applications. Their derivatives demonstrate a broad range of pharmacological activities such as antimicrobial, antidiabetic, analgesic, and anticancer activities [24–26]. Several anticancer agents containing urea and thiourea functional groups reached clinical phases [27] such as Tandutinib and Enzalutamide, respectively (Figure 1). Moreover, novel series of benzimidazole-thiourea [28] and benzimidazole-urea [29] derivatives were synthesized and they exhibited potent antiproliferative activity against a group of human tumor cells compared to standard drugs. Hybrids of urea and benzimidazole showed potent anticancer activity and some derivatives are already in the drug market such as Glasdegib [30] (Figure 1).

According to the World Health Organization (WHO), the number of people diagnosed with cancer increased from 10 million in 2000 to 19.3 million in 2020. Breast cancer (BC) is the world's most diagnosed cancer as reported by International Agency for Research on Cancer (IARC) in 2020 (WHO, 2021) with metastasis and drug

Figure 1: Potent anticancer agents.

resistance being the main challenge for successful treatments [31]. Moreover, the drug resistance of cancer cells exerts more pressure to look for new effective chemotherapeutic agents. BC is subtyped or classified based on their genotypic differences and metastasis characteristics. The toxicity of the drug against different types of BC cells differs significantly due to variation in the metabolic outcome of their genotype [32,33].

Apoptosis is an important biological event that takes place in multicellular organisms to remove unwanted or damaged cells [34]. Apoptosis has been extensively used for therapeutic applications and biological studies and huge effort has been dedicated to the discovery of apoptosis-inducing molecules that may have antitumor potential [35].

Based on the biological activity profile of benzimidazole derivatives and thiourea and urea derivatives, the current study is aimed to synthesize novel thiourea- and urea-benzimidazole derivatives and evaluate their anticancer activity against two different cancer cell lines (MDA-MB-231^{ER(-)/PR(-)} and MCF-7^{ER(+)/PR(+)}) with different genotypic features.

2 Experimental methods

2.1 Materials and methods

Melting points were determined in open capillary tubes using the Sanyo Gallenkamp MPD 350-BM 3.5 Melting Point apparatus (UK), and are uncorrected. FT-IR spectra

were recorded in a Thermo Nicolet Nexus 470 FT-IR spectrophotometer (USA). ¹H NMR and ¹³C NMR spectra were recorded at room temperature in CDCl₃ or DMSO-*d*₆ as a solvent using Varian-400 MHz (USA). Solvent peaks (CDCl₃: 7.26 [D] and 77.2 [C] ppm and DMSO-*d*₆: 2.50 [D] and 39.7 [C] ppm) were used as internal references. The assignment of chemical shifts is based on standard NMR experiments (¹H and ¹³C). TLC analyses on silica F254 and detection by UV light at 254 nm were performed. High resolution mass spectra (HRMS) were obtained (in positive or negative mode) using the electrospray ion trap (ESI) technique by collision-induced dissociation on a Bruker APEX-4 (7-Tesla) instrument. Column chromatography was performed on silica Gel 60 (230 mesh). Chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (1-Boc-ethylenediamine, 1-Boc-piperazine, 2-chlorobenzimidazole, and 4M HCl in dioxane) and Acros Organics, USA (2-chlorobenzimidazole, isothiocyanates, and isocyanates) and used without further purification. 2-(1-Piperazino)-benzimidazole.2HCl (**1**) and N1-(benzimidazol-2-yl)ethane-1,2-diamine.2HCl (**6**) were prepared according to reported literature procedures [35–38].

2.2 Synthesis

2.2.1 General synthesis of benzimidazole-thiourea derivatives (4a–j, 7b) and benzimidazole-urea derivatives (5b–e, 8b)

To a stirred solution of compounds **1** or **6** (1.1 mmol) in dry acetonitrile (10 mL), was added excess triethylamine

(4 mmol) and the corresponding isothiocyanatobenzene **2a–j** or isocyanatobenzene **3b–e** (1.0 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under vacuum, dissolved in ethyl acetate (20 mL) and then washed with water. The organic layer was dried over anhydrous sodium sulfate and the solution was concentrated. The crude product was filtered, dried, and recrystallized from ethyl acetate/hexane.

2.2.2 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-phenylpiperazine-1-carbothioamide (**4a**)

Off white solid (0.53 g, 86%); mp 229–231°C; R_f = 0.74 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,399 (NH), 1,530 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 9.49 (s, 1H), 7.32–7.28 (m, 4H), 7.27–7.14 (m, 2H), 7.15–7.10 (m, 1H), 7.07–7.15 (m, 2H), 4.11–4.09 (m, 4H), 3.68–3.66 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 182.0 (C=S), 155.7, 141.4, 137.5, 128.5, 125.8, 124.9, 120.5, 113, 47.7, 46.02. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{20}\text{N}_5\text{S}$ [M + H] 338.14394, found 338.14339.

2.2.3 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(*p*-tolyl)piperazine-1-carbothioamide (**4b**)

Off white solid (0.28 g, 72%); mp 231–233°C; R_f = 0.72 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,430 (NH), 1,523 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.53 (s, 1H), 9.37 (s, 1H), 7.33 (m, 2H), 7.27–7.14 (m, 2H), 7.13–7.06 (m, 2H), 6.95 (m, 2H), 4.16–3.95 (m, 4H), 3.67–3.47 (m, 4H), 2.28 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 182.1 (C=S), 156.0, 138.7, 134.1, 130.8, 129.0, 126.2, 126.0, 120.3, 47.6, 46.0, 21.0. HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{22}\text{N}_5\text{S}$ [M + H] 352.15959, found 352.15904.

2.2.4 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-methoxyphenyl)piperazine-1-carbothioamide (**4c**)

White solid (0.38 g, 57%); mp 285–287°C; R_f = 0.77 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,413 (NH), 1,527 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.46 (s, 1H), 9.31 (s, 1H), 7.16 (m, 4H), 6.86 (m, 4H), 4.13–3.94 (m, 4H), 3.73 (s, 3H), 3.64–3.52 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 182.2 (C=S), 156.9, 156.0, 134.2, 127.9, 126.3, 120.2, 116.5, 113.6, 55.6, 47.6, 46.10. HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{22}\text{N}_5\text{OS}$ [M + H] 368.15451, found 368.15491.

2.2.5 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(3-chlorophenyl)piperazine-1-carbothioamide (**4d**)

Off white solid (0.20 g, 74%); mp 248–250°C; R_f = 0.74 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,428 (NH), 1,529 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.56 (s, 1H), 9.54 (s, 1H), 7.46 (m, 1H), 7.37–7.27 (m, 2H), 7.25–7.20 (m, 2H), 7.17 (m, 1H), 6.95 (m, 2H), 4.11–4.02 (m, 4H), 3.69–3.54 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 181.7 (C=S), 155.9, 142.9, 139.0, 132.6, 130.0, 125.1, 124.4, 123.9, 120.3, 113.0, 47.8, 46.9. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{ClN}_5\text{S}$ [M + H] 372.10497, found 372.10442.

2.2.6 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-chlorophenyl)piperazine-1-carbothioamide (**4e**)

Off white solid (0.53 g, 79%); mp 246–248°C; R_f = 0.71 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,399 (NH), 1,524 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.48 (s, 1H), 9.50 (s, 1H), 7.63–7.40 (m, 1H), 7.34 (m, 3H), 7.22 (m, 2H), 6.94 (m, 2H), 4.17–3.98 (m, 4H), 3.70–3.51 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 181.8 (C=S), 156.1, 140.4, 128.8, 128.3, 127.4, 125.5, 119.6, 115.6, 47.8, 46.0. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{ClN}_5\text{S}$ [M + H] 372.10497, found 372.10465.

2.2.7 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-nitrophenyl)piperazine-1-carbothioamide (**4f**)

Yellow solid (0.25 g, 91%); mp 204–206°C; R_f = 0.84 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,435 (NH), 1,530 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.63 (s, 1H), 9.96 (s, 1H), 8.18 (d, J = 9.2 Hz, 2H), 7.63 (d, J = 9.1 Hz, 2H), 7.23 (m, 2H), 6.96 (m, 2H), 4.13–4.06 (m, 4H), 3.68–3.60 (m, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 181.3 (C=S), 155.7, 148.2, 142.6, 128.8, 124.4, 123.2, 120.4, 113.4, 48.3, 46.0. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{N}_6\text{O}_2\text{S}$ [M + H] 383.12902, found 383.12877.

2.2.8 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(2-fluorophenyl)piperazine-1-carbothioamide (**4g**)

Yellowish solid (0.21 g, 80%); mp 268–270°C; R_f = 0.8 (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,458 (NH), 1,528 (C=S); ^1H NMR (400 MHz, DMSO- d_6) δ 11.82 (s, 1H), 9.30 (s, 1H), 7.32–7.21 (m, 5H), 7.21–7.13 (m, 1H), 7.04–6.94 (m, 2H), 4.17–3.96 (m, 4H), 3.74–3.58 (m, 4H).

^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 182.7 (C=S), 158.1 (d, $^1J_{\text{C-F}} = 246.0$ Hz), 151.9, 136.9, 130.6 (d, $^3J_{\text{C-F}} = 3.5$ Hz), 129.2 (d, $^2J_{\text{C-F}} = 11.9$ Hz), 128.2 (d, $^3J_{\text{C-F}} = 7.2$ Hz), 124.5 (d, $^4J_{\text{C-F}} = 3.9$ Hz), 121.1, 116.2 (d, $^2J_{\text{C-F}} = 20$ Hz), 112.5, 47.5, 46.0. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{FN}_5\text{S}$ [M + H] 356.13452, found 356.13397.

2.2.9 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(3-fluorophenyl) piperazine-1-carbothioamide (4h)

White solid (0.52 g, 80%); mp 229–231°C; $R_f = 0.71$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,410 (NH), 1,535 (C=S); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.58 (s, 1H), 9.55 (s, 1H), 7.33 (td, $J = 8.2, 6.8$ Hz, 1H), 7.26 (dt, $J = 11.2, 2.3$ Hz, 1H), 7.24–7.19 (m, 2H), 7.16 (m, 1H), 7.00–6.89 (m, 3H), 4.10–4.04 (m, 4H), 3.66–3.58 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 181.7 (C=S), 161.5 (d, $^1J_{\text{C-F}} = 241$ Hz), 155.6, 143.3 (d, $^3J_{\text{C-F}} = 4.5$ Hz), 138.2, 129.9 (d, $^4J_{\text{C-F}} = 3.1$ Hz), 121.1, 121.0 (d, $^3J_{\text{C-F}} = 2.9$ Hz), 120.5, 112.2 (d, $^2J_{\text{C-F}} = 24$ Hz), 110.1 (d, $^2J_{\text{C-F}} = 21$ Hz), 47.9, 46.1. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{FN}_5\text{S}$ [M + H] 356.13452, found 356.13407.

2.2.10 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-fluorophenyl) piperazine-1-carbothioamide (4i)

Beige solid (0.64 g, 98%); mp 248–250°C; $R_f = 0.78$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,193 (NH), 1,569 (C=S); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.48 (s, 1H), 9.44 (s, 1H), 7.31 (m, 2H), 7.22 (m, 2H), 7.14 (m, 2H), 6.94 (m, 2H), 4.28–3.89 (m, 4H), 3.79–3.50 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 182.2 (C=S), 159.7 (d, $^1J_{\text{C-F}} = 240$ Hz), 155.3, 138.1, 137.7 (d, $^4J_{\text{C-F}} = 3$ Hz), 128.2 (d, $^3J_{\text{C-F}} = 8.3$ Hz), 123.9, 120.6, 115.1 (d, $^2J_{\text{C-F}} = 22$ Hz), 47.5, 46.0. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{FN}_5\text{S}$ [M + H] 356.13452, found 356.13421.

2.2.11 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-(trifluoromethyl)phenyl) piperazine-1-carbothioamide (4j)

White solid (0.248 g, 84%); mp 242–246°C; $R_f = 0.78$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,423 (NH), 1,529 (C=S); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.73 (s, 1H), 8.95 (s, 1H), 7.66 (d, $J = 8.5$ Hz, 1H), 7.58 (d, $J = 8.5$ Hz, 1H), 7.27 (dd, $J = 5.8, 3.2$ Hz, 1H), 7.02 (dd, $J = 5.7, 3.2$ Hz, 1H), 4.20–4.00 (m, 4H), 3.76–3.58 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 181.8 (C=S), 156.0, 145.3, 129.0, 126.3, 125.6,

124.8, 124.1, 123.8, 123.6, 120.3, 48.0, 46.0. HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{18}\text{F}_3\text{N}_5\text{S}$ [M + H] 406.13133, found 406.13162.

2.2.12 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(*p*-tolyl) piperazine-1-carboxamide (5b)

Beige solid (0.34 g, 55%); mp 298–300°C; $R_f = 0.74$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,407 (NH), 1,637 (C=O); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.46 (s, 1H), 8.55 (s, 1H), 7.34 (m, 2H), 7.21 (s, 2H), 7.06 (m, 2H), 7.00–6.85 (m, 2H), 3.59 (dd, $J = 7.1, 3.4$ Hz, 4H), 3.53 (dd, $J = 7.1, 3.6$ Hz, 4H), 2.23 (s, 3H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 156.4 (C=O), 155.5, 138.2, 137.7, 131.1, 129.6, 129.2, 120.3, 118.6, 115.5, 109.5, 46.4, 43.6, 20.8. HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{22}\text{N}_5\text{O}$ [M + H] 336.18244, found 336.18256.

2.2.13 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-methoxyphenyl) piperazine-1-carboxamide (5c)

Off white solid (0.16 g, 63%); mp 297–299°C; $R_f = 0.75$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,467 (NH), 1,634 (C=O); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.55 (s, 1H), 8.48 (s, 1H), 7.39–7.31 (m, 2H), 7.21 (m, 2H), 6.94 (m, 2H), 6.88–6.80 (m, 2H), 3.70 (s, 3H), 3.61–3.55 (m, 4H), 3.55–3.49 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 156.3 (C=O), 155.7, 154.9, 133.7, 133.3, 122.0, 120.3, 114.4, 114.0, 55.5, 46.4, 43.5. HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{22}\text{N}_5\text{O}_2$ [M + H] 352.17735, found 352.17744.

2.2.14 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(3-chlorophenyl) piperazine-1-carboxamide (5d)

White solid (0.41 g, 63%); mp 287–289°C; $R_f = 0.68$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1}) 3,434 (NH), 1,636 (C=O); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.46 (s, 1H, NH), 8.82 (s, 1H, NH), 7.67 (s, 1H), 7.42 (d, $J = 8.8$ Hz, 1H), 7.27 (t, $J = 8.4$ Hz, 1H), 7.21 (m, 2H), 6.99 (d, $J = 8.4$ Hz, 1H), 6.94 (m, 2H), 3.63–3.57 (m, 4H), 3.56–3.50 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 156.4 (C=O), 155.5, 142.5, 136.2, 135.6, 131.1, 129.9, 129.6, 129.2, 120.3.1, 118.6, 46.3, 43.6. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{ClN}_5\text{O}$ [M + H] 356.12781, found 356.12726.

2.2.15 4-(1*H*-Benzo[d]imidazol-2-yl)-*N*-(4-chlorophenyl) piperazine-1-carboxamide (5e)

Off white solid ($m = 0.304$ g, 50%); mp 287–289°C; $R_f = 0.70$ (9:1 dichloromethane/methanol), IR (KBr, cm^{-1})

3,259 (NH), 1,642 (C=O); ^1H NMR (400 MHz, DMSO- d_6) δ 11.46 (s, 1H), 8.77 (s, 1H), 7.39 (d, J = 8.9 Hz, 2H), 7.29 (d, J = 8.9 Hz, 1H), 7.21 (m, 2H), 7.08–6.75 (m, 2H), 3.59 (dd, J = 7.1, 3.6 Hz, 4H), 3.53 (dd, J = 6.8, 3.7 Hz, 4H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 155.3, 139.2, 138.1, 137.6, 129.1, 128.3, 126.1, 120.4, 111.7, 46.4, 43.4. HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{ClN}_5\text{O}$ [$\text{M} + \text{H}$] 356.12781, found 356.12726.

2.2.16 1-(2-(1*H*-Benzo[d]imidazol-2-ylamino)ethyl)-3-p-tolylthiourea (7b)

Beige solid (m = 0.182 g, 97%); mp 165–167°C, IR (KBr, cm^{-1}) 3,271 (NH), 1,684 (C=S); ^1H NMR (400 MHz, Methanol- d_4) δ 9.32 (s, 1H), 7.45 (s, 2H), 7.33–7.31 (m, 2H), 7.24–7.17 (m, 2H), 7.12–7.10 (m, 2H), 7.04–7.02 (m, 2H), 3.94–3.87 (m, 2H), 3.64–3.61 (m, 2H), 2.27 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 181.7 (C=S), 151.7, 136.1, 135.0, 131.9, 130.1, 124.5, 122.7, 122.3, 42.9, 42.4. HRMS (ESI): m/z calcd for $\text{C}_{17}\text{H}_{20}\text{N}_5\text{S}$ [$\text{M} + \text{H}$] 326.14394, found 326.14339.

2.2.17 1-(2-(1*H*-Benzo[d]imidazol-2-ylamino)ethyl)-3-p-tolylurea (8b)

Beige solid (m = 0.069 g, 77%); mp 100–102°C; IR (KBr, cm^{-1}) 3,370 (NH), 1,705 (C=O); ^1H NMR (400 MHz, CD_3OD) δ 8.33 (s, 1H), 7.38–7.31 (m, 2H), 7.26–7.19 (m, 2H), 7.18–7.11 (m, 2H), 7.10–7.01 (m, 2H), 6.42 (s, 1H), 3.57–3.54 (m, 2H), 3.50–3.47 (m, 2H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 157.5 (C=O), 151.2, 136.5, 132.1, 130.7, 128.9, 123.0, 119.5, 111.0, 43.4, 38.5. HRMS (ESI): m/z calcd for $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}$ [$\text{M} + \text{H}$] 310.16679, found 310.16639.

2.3 Cytotoxicity assay

The cytotoxic activity of the synthesized compounds was assessed in human BC cell lines. MDA-MB-231^{ER(-)/PR(-)} and MCF-7^{ER(+)/PR(+)} cancer cells were obtained from German Collection of Cell Cultures (DSMZ, Germany). Cells were

X					
S			O		
Code	R	%Yield	Code	R	%Yield
4a	H	86			
4b	4-CH ₃	72	5b	4-CH ₃	55
4c	4-OCH ₃	57	5c	4-OCH ₃	63
4d	3-Cl	74	5d	3-Cl	63
4e	4-Cl	79	5e	4-Cl	50
4f	4-NO ₂	91	8b	4-CH ₃	77
4g	2-F	80			
4h	3-F	80			
4i	4-F	98			
4j	4-CF ₃	84			
7b	4-CH ₃	97			

Scheme 1: Synthesis of benzimidazole thiourea (4a–j, 7b) and benzimidazole urea (5b–e, 8b) derivatives.

Table 1: The concentration with 50% inhibition (IC_{50} in μM) of derivatives **7b** and **5d** against breast cancer cell lines, MCF7 and MDA-MB-231

	MCF-7 (IC_{50} in μM)	MDA-MB-231 (IC_{50} in μM)
7b	25.8 ± 0.5	54.3 ± 0.09
5d	48.3 ± 0.8	89.5 ± 0.1

cultured in high glucose DMEM (UFC Biotech, Saudi Arabia) containing 1% of antibiotic (Penicillin–Streptomycin, UFC Biotech, Saudi Arabia) supplemented with 10% of fetal bovine serum (GIBCO, USA). These cells were maintained at 37°C and in 5% of CO₂. Cells were seeded in 96-well plates (5×10^4 cells/well) and treated with different concentrations for 24 h. Afterwards, the medium containing the compounds was removed. Subsequently, MTT solution (5 mg mL^{-1}) was added to each well and incubated for 2 h. Then, absorbance was read in a microplate reader (Microplate Reader, Biotek) using 550 nm wavelengths. The control received only DMSO

of 0.1%. IC_{50} values were determined using the OriginPro 8.5 software.

2.4 Light microscopy

To investigate the effects of active compounds on the morphology of MCF-7, the cells were seeded in DMEM. The cells were treated with $2 \times IC_{50}$ value for 24 h, and imaged using a light microscope (Leica, Germany) at 200× magnification.

2.5 4',6-Diamidino-2-phenylindole (DAPI) staining assay

DAPI staining was carried according to ref. [39] with modifications. Cells were cultured in a 24-well culture

Figure 2: (a) Cytotoxic activity of **7b** on MCF-7 and MDA-MB-231 cells using MTT assay; (b) control; and (c) cells treated with **7b** showing shrinkage and detachment from the surface (24 h).

plate (Nist, China) for 24 h. After incubation with $2 \times \text{IC}_{50}$ of **7b** and **5d** for 24 h, cells were washed with PBS, fixed with ice cold methanol for 5 min and then were again washed with PBS. After washing, the cells were stained with DAPI ($1 \mu\text{g mL}^{-1}$) and incubated in dark for 30 min. Methanol was used as a control. The cells were photographed using a fluorescence microscope (Evos, USA).

2.6 Acridine orange/ethidium bromide (AO/EB) staining

MCF-7 cells were incubated with **7b** and **5d** ($2 \times \text{IC}_{50}$) as described in the Section 2.5 for 24 h, then stained with AO/EB ($2 \mu\text{g mL}^{-1}$) for 10 min, and were viewed using fluorescence microscopy.

2.7 Caspases-3/7

The assessment of caspase-3/7 was carried out based on the manufacturer's manual. Briefly, the cells were cultured in a 24-well culture plate (Nist, China) for 24 h. The cells

were treated as described in the Section 2.5. After treatment, $1 \mu\text{g mL}^{-1}$ of caspase-3/7 reagent (Invitrogen, USA) was added and shaken for 30 s and incubated in dark for 30 min. Methanol was used as a control. The cells were photographed using a fluorescence microscope.

3 Results and discussion

The synthesis of thiourea and urea derivatives of benzimidazole is outlined in Scheme 1. The synthesis of target thiourea **4a–j** and urea **5b–e** was achieved by reacting 2-(piperazin-1-yl)-1*H*-benzo[*d*]imidazole hydrochloride **1** [30,31] with substituted isothiocyanates **2a–j** and isocyanates **3b–e**, respectively, in the presence of an excess of triethylamine to obtain good yields.

Similarly, two derivatives thiourea **7b** and urea **8b** were prepared by reacting N1-(1*H*-benzo[*d*]imidazol-2-yl)ethane-1,2-diamine hydrochloride **6** [38] with isothiocyanates **2b** and isocyanates **3b**, respectively. The purity of the prepared compound is confirmed by recrystallization, its melting point, chromatographic technique, and spectroscopy data.

Figure 3: (a) Cytotoxic activity of **5d** on MCF-7 and MDA-MB-231 cells using MTT assay; (b) control; and (c) cells treated with **5d** showing shrinkage and detachment from the surface (24 h).

Figure 4: Fluorescence image of MCF-7 cells treated with DAPI, AO/EB, or caspase-3/7 reagent after 24 h of treatment with **7b**. DAPI staining shows chromatin condensation and nuclear fragmentation as indicated with arrows. (a) Untreated cells and (b) treated cells. AO/EB staining: (c) untreated cells and (d) treated cells. Live cells are stained evenly green, whereas apoptotic cells are characterized by shiny green and yellow-orange staining due to loss of membrane integrity and chromatin condensation. Caspase-3/7 activation: (e) untreated cells and (f) treated cells. Caspase-3/7 activation was demonstrated by green fluorescence. Magnification $\times 200$.

The newly synthesized compounds (**4a–j**, **7b**) and (**5b–e**, **8b**) were characterized by MS, ^1H and ^{13}C NMR, and IR spectral data. These data, detailed in the experimental part, are consistent with the suggested structures. The ^{13}C NMR data revealed the presence of a signal between 181.1 and 182.7 ppm for compounds (**4a–j**, **7b**) and between 155.3 and 157.5 ppm for compounds (**5b–e**, **8b**). This signal corresponds to quaternary carbons $\text{C}=\text{S}$ and $\text{C}=\text{O}$, respectively. In the IR spectra, absorption bands associated with $\text{C}=\text{S}$ and $\text{C}=\text{O}$ stretching vibrations are appearing between 1,523 and 1,596 cm^{-1} for compounds (**4a–j**, **7b**) and between 1,634 and 1,642 cm^{-1} for compounds (**5b–e**, **8b**), respectively.

The HRMS of the synthesized compounds are in good agreement with the calculated values.

The diversity of the proposed compounds in Scheme 1 is evident from the different functional groups (urea and thiourea), substitution on the phenyl ring including activating and deactivating groups, and also from using the piperazine and ethylenediamine as spacers. These structure variations can help investigating the structure–activity relationship.

The newly synthesized thiourea **4a–j** and urea **5b–e** were investigated for cytotoxicity using MTT cell viability assay in two human BC cell lines, MCF-7 and MDA-MB-

231. As shown in Figures 2 and 3, only the compounds **7b** and **5d** were cytotoxic to the tested cancer cell lines. For the remaining compounds, the IC_{50} values were not calculated because they were higher than $1,000 \mu\text{g mL}^{-1}$. However, the cytotoxicity of compounds **7b** and **5d** was higher in MCF-7 with IC_{50} values of 25.8 and $48.3 \mu\text{M}$, respectively, as compared with their activity on MDA-MB-231 cells. The IC_{50} values for **7b** and **5d** compounds were 54.3 and $89.5 \mu\text{M}$, respectively, on MDA-MB-231 cells (Table 1).

Morphological alternations in the cells were observed using phase contrast microscope after incubation with **7b** and **5d** for 24 h. Cells in the control group retained the

morphological form, most of which were adherent to the surface, tightly packed, and distinctively monolayer. Exposure of the BC cells to **7b** and **5d** compounds led to rounding, shrinkage of the cells, loss of contact, and debris formation (Figures 2b and c and 3b and c).

The potential of inducing apoptosis by the **7b** and **5d** was assessed by DAPI staining. The results indicated that the number of apoptotic cells was higher in **7b**- and **5d**-treated cells than DMSO control. The changes that took place in MCF-7 cells as a result of **7b** and **5d** treatments are represented in Figures 4a and b and 5a and b. The treatment resulted in chromatin condensation and nuclear fragmentation which are apoptotic indicators.

Figure 5: Fluorescence image of MCF-7 cells treated with DAPI, AO/EB, or caspase-3/7 reagent after 24 h of treatment with **5d**. DAPI staining shows chromatin condensation and nuclear fragmentation as indicated with arrows: (a) untreated cells and (b) treated cells. AO/EB staining: (c) untreated cells and (d) treated cells. Live cells are stained evenly green, whereas apoptotic cells are characterized by shiny green and yellow-orange staining due to loss of membrane integrity and chromatin condensation. Caspase-3/7 activation: (e) untreated cells and (f) treated cells. Caspase-3/7 activation was demonstrated by green fluorescence. Magnification $\times 200$.

The apoptotic morphology was detected by AO/EB fluorescent staining of MCF-7 cells incubated with **7b** and **5d** compounds. As evident in Figures 4c and d and 5c and d, nuclei stained with green color demonstrate live cells, while greenish yellow and orange-red nuclei show apoptotic cells. The red color shows dead cells. The negative control (untreated cells) cells appeared bright green.

In apoptotic cells, caspase-3/7 activity cleaves the substrate, releasing a DNA dye, which moves to the nucleus and binds with DNA that gives shiny green fluorescence. The treated cells (Figures 4e and f and 5e and f) showed an increase in the caspase-3/7 activity after 24 h of exposure to **7b** and **5d**, indicating that the apoptosis induced is caspase-3/7 dependent.

Apoptosis is a highly organized physiological mechanism to destroy abnormal cells [40] and it is an attractive endpoint in anticancer agents' research. It is induced by many effective anticancer agents [41]. A variety of compounds have been known to induce apoptosis in various human cancer cells. So it is important to screen for apoptotic inducers from synthetic anticancer compounds. There are many ways of detecting apoptosis. Detection of apoptosis using fluorescence microscope has many advantages over other methods because it does not include an enzymatic reaction that is affected by many variables such as pH, temperature, and composition buffers [42]. Single and double staining methods for detecting apoptosis (AO/EB), produce reliable and reproducible results. The results of the apoptotic cell identified by the AO/EB method are highly reproducible [43].

Induction of apoptosis is often linked to the activation of caspases. Incubation of MCF-7 cells with **7b**- and **5d**-induced caspase-3/7 activity is evident by a bright green fluorescence in treated cells after 24 h. Thus, apoptosis induced by **7b** and **5d** was caspase dependent. Both pathways, intrinsic and extrinsic pathways, activate downstream effector caspases mainly caspase-3 and caspase-7 [44]. Apoptosis is the main target in treatment strategies of cancer. Anticancer compounds that induce apoptosis are promising candidate for cancer therapy. Further studies are required to assess other pathways for apoptosis. These data provide a scientific foundation for the discovery and development of novel anticancer compounds from benzimidazole thiourea and benzimidazole urea derivatives.

4 Conclusion

In summary, we have designed and synthesized a series of 16 novel compounds (**4a-j**), (**5b-e**), **7b**, and **8b** that

contain benzimidazole, piperazine, ethylenediamine, and urea or thiourea moieties. These compounds were characterized by using suitable spectroscopic techniques including ^1H and ^{13}C NMR, IR, and high-resolution mass spectrometry. *In vitro* anticancer assay against breast cancer cell lines, MCF-7 and MDA-MB-231, revealed that the cytotoxicity of compounds **7b** and **5d** were higher in MCF7 with IC_{50} values of 25.8 and 48.3 μM , respectively, as compared with their activity in MDA-MB-231 cells. These results could be a lead to prepare new benzimidazole derivatives with potent anti-cancer activity.

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