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Design, Molecular Docking study, Synthesis, and Preliminary Cytotoxic Evaluation of Some New 5-Methoxy-2-mercaptobenzimidazole Derivatives

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ABSTRACT

Benzimidazole scaffolds are biologically and therapeutically useful chemical motifs against several diseases. Several derivatives are already known for benzimidazole, one of the most important derivatives known is 2-mercaptobenzimidazole with numerous biological activities were reported *viz.* antimicrobial, antiviral, anti-tumor and anti-inflammatory. Thus, several 2-mercaptobenzimidazole derivatives were designed and directly prepared through *S*-alkylation with four different para-substituted 1-bromomethyl benzene and *N*-alkylation with 1-(2-chloroethyl)piperidine, 1-(2-chloroethyl)-4-methylpiperazine and 1-(2-chloroethyl)-4-morpholine moieties with potential cytotoxic activities against breast cancer. Prior to the synthesis of the target compounds, docking studies were conducted which showed good docking scores compared to the standard raloxifene against estrogen receptor alpha (ERα), which is considered one of the main molecular targets in breast cancer pathogenesis. The synthesis of the target compounds 14 a-d was successful, and their structures were confirmed with FT-IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS analysis. The in-vitro cytotoxicity assay (MTT assay) demonstrated that compound 14c possesses excellent cytotoxic effects towards breast cancer cell line (MDA-MB-231: IC_{50}: 24.78± 1.02 µM), compared to standard raloxifene with an IC_{50}: 26.73 µM. From the docking study, it was concluded that piperidine, methyl-piperazine and morpholine moiety successfully bind tightly to alpha estrogen receptors by making numerous interaction modes.

1. Introduction

Cancer is one of the world's most serious clinical problems, involving the uncontrolled proliferation of abnormal cells capable of dedifferentiation, invasion, and metastasis via the bloodstream or lymphatic system [1]. Its incidence is increasing in both developing and developed countries, and it is regarded as one of the most fatal diseases in human history [2, 3]. In men, the highest percentages of cancer types occur in the prostate, lung and bronchus, colon and rectum, and urinary bladder, respectively. Women are more likely to develop cancer in the breast, lung and bronchus, colon and rectum, uterine corpus, and thyroid. This data shows that prostate and breast cancer account for a large proportion of cancer in men and women, respectively [4]. Breast cancer is a histologic diagnosis made according to standardized pathologic criteria [5]. The most common breast cancer histology is invasive ductal carcinoma (50%-75% of patients), followed by invasive lobular carcinoma (5%-15%of patients), with mixed ductal/ lobular carcinomas and other rarer histologies making up the remainder of patients [6]. Two main molecular targets in breast cancer pathogenesis have been identified. One is estrogen receptor alpha (ERα), which is expressed in approximately 70%of invasive breast cancers. ERα is a steroid hormone receptor and a transcription factor that, when activated by estrogen, activates oncogenic growth pathways in breast cancer cells. Expression of the closely

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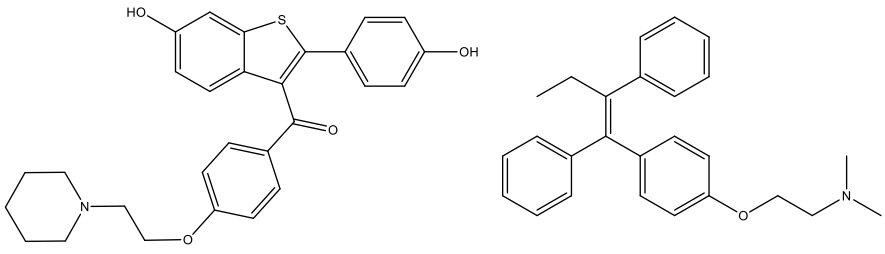
related steroid hormone progesterone receptor (PR) is also a marker of ERα signaling [7, 8]. The second main molecular target is epidermal growth factor 2 (*ERBB2*, formerly *HER2* or *HER2/neu*), a transmembrane receptor tyrosine kinase in the epidermal growth factor receptor family that is amplified or overexpressed in approximately 20% of breast cancers, and is associated with poor prognosis in the absence of systemic therapy [9, 10]. Tamoxifen and raloxifene, have complex patterns of activity in estrogen-responsive tissues, acting as so-called selective estrogen receptor modulators (SERMs) (Figure 1). Raloxifene, a SERM with a benzothiophene backbone that is prescribed for prevention of osteoporosis and associated with favorable agonist-like action on lipid metabolism, has shown to retain 76% of the effectiveness of tamoxifen at reducing invasive breast cancer incidence with a significantly lower incidence of endometrial cancer in the Study of Tamoxifen and Raloxifene (STAR) prevention trial, but is not effective in patients resistant to tamoxifen [11].



Raloxifen (1) **Tamoxifen (2)**

Fig. 1. Structures of raloxifene and tamoxifen

Medicinal chemists pursue in the design and synthesis of new anticancer drugs that possess therapeutic effects with less adverse reactions and resistance is an ongoing challenge. Heterocyclic compounds in particular are important in the field of medicine, biochemistry and many other fields of science [12]. Simple heterocyclic scaffold is considered particularly important for obtaining new drugs with vast biological activities. 2-mercaptobenzimidazole (2-MBI) is an organosulfur compound with wide range of pharmacological properties were reported, such as antimicrobial, antihistamine, analgesic, and anticonvulsant activities [13]. 2-MBI and their derivatives such as 5-Methoxy-2-mercapto benzimidazole (Figure 2), have been chemically modified quite significantly, with a positive outcome on their biological activity. Recent work published new 2-MBI derivatives with cytotoxic activity against Epidermal Growth Factor Receptor (EGFR) [14]. Moreover, it was reported that chemical modification of 2-MBI through Schiff base formation leads to new derivatives with cytotoxic activities against HCT-116 cancer cell Line [15].

2-Mercaptobenzimidazole (3) **5-methoxy-1H-benzo[d]imidazole-2-thiol (4)**

Fig. 2. Structure of 2-mercaptobenzimidazole (2-MBI, 3) and 5-methoxy-1H-benzo[d]imidazole-2-thiol (4).

2. Materials and methods

2.1. Materials

Piperidine,1-methyl piperazine, morpholine, 5-methoxy-1H-benzo[d] imidazole-2-thiol, 1-bromo-2-chloroethane, 4-chloromethyl phenyl acetate, 1-(bromomethyl)-4-chlorobenzene, 1-(bromomethyl)-benzene, and ethanethiol were purchased from Heowns (China) and Sigma–Aldrich (Germany). All chemicals are of analytical grade, and they were used as received without further purification.

2.2. Characterization of intermediate Compounds and Compounds (14 a -d)

The progression and ending of reactions were monitoring and evaluated using UV light and thin-layer chromatography (TLC) on Merck silica gel 60F254, the solvent system that used as mobile phase was methanol: dichloromethane (1:10), determination of functional groups in compounds was done by the Infrared

spectroscopy (FT-IR) spectra which were recorded on FT-IR spectrophotometer/ Shimadzu, Japan, supplied by Specac® Quest ATR (diamond)- UK (College of Pharmacy, University of Baghdad), ^{1}H-NMR (^{1}HNMR spectroscopy (400 MHz) (Bruker Avance II) , C^{13} NMR [13C-NMR spectroscopy (100 MHZ) (Bruker Avance II)], Mass Spectroscopy [(Electrospray Ionization) compact™ ESI QTOF Mass Spectrometer Bruker Daltonics, Germany] in the BU-Ali Research center, Mashhad University of Medical Science, Mashhad, Iran .

In Silico Molecular docking was carried out using Maestro software (Schrödinger, version 2022-1), and ΔG (kcal/mol) as a docking scoring function, the protein used (7kbs: Estrogen Receptor Alpha Ligand Binding Domain in Complex with Raloxifene) is downloaded from protein data bank [16].

Cytotoxicity assay was performed at the BU-Ali Research center, Mashhad University of Medical Science, Mashhad, Iran. HUVEC (a human normal cell line), SK-OV-3 (a human ovarian cancer cell line) and MDA-MB-231 (a human breast cancer cell line) were purchased from National Cell Bank of Iran (Pasteur Institute, Iran). Cells were grown in RPMI-1640 (Gibco) with 10% FBS (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained at 37 °C under humidified air containing 5% CO_2 and were passaged using trypsin/EDTA (Gibco) and phosphate- buffered saline (PBS) solution. Culturing media and conditions used to grow the cells as 3D colonies was the same as monolayer cell culture, the concentrations used were 6.25, 12.5, 25, 50, and 100 µM, and the exposure time was 72 hr.

3. Chemical synthesis

The target compounds were synthesized by multi-step reactions, as shown in Scheme 1.

3.1. Chemical synthesis (procedure A) of 1-(2-chloroethyl) cyclic amine (Compound 7 a-c)

To a mixture of (0.01 mole) of cyclic amines (piperidine, 1-methyl piperazine and morpholine respectively) in anhydrous DMF (10 ml) and anhydrous potassium carbonate (0.011 mole), 1-bromo-2-chloroethane (0.01 mole) was added. The mixture was stirred for 24 hours and water (100 ml) was added. Termination of reaction was approved by TLC. Then the mixture was extracted with ethyl acetate (3 × 50 ml) and organic layers were combined, washed with brine (50 ml), and dried over anhydrous sodium sulfate. The mixture was filtered and the solvent was removed by rotary evaporation under reduced pressure and the crude amines obtained as a liquid were converted to the hydrochloride salt and recrystallized from ethanol to obtain compounds 7a, 7b and 7c [17].

1-(2-Chloroethyl)piperidine hydrochloride (7a) was obtained using the general procedure A (88%) as a yellow powder. IR (FT) λ max cm^{-1}: 2927, 1442, 763.

1-(2-Chloroethyl)-4-methylpiperazine dihydrochloride (7b) was obtained using the general procedure A (85%) as a white-yellow powder. IR (FT) λ max cm^{-1}: 2970, 1450-1384, 678.

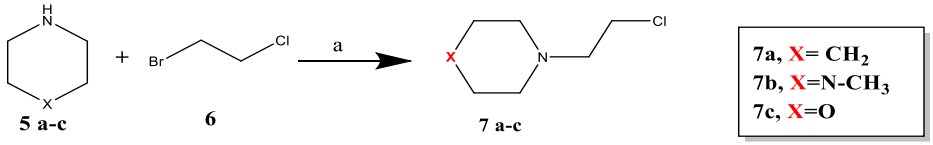
1-(2-chloroethyl) morpholine hydrochloride (7c) was obtained using the general procedure A (89%) as a yellow powder. IR (FT) λ max cm^{-1}: 2927, 1442, 763.

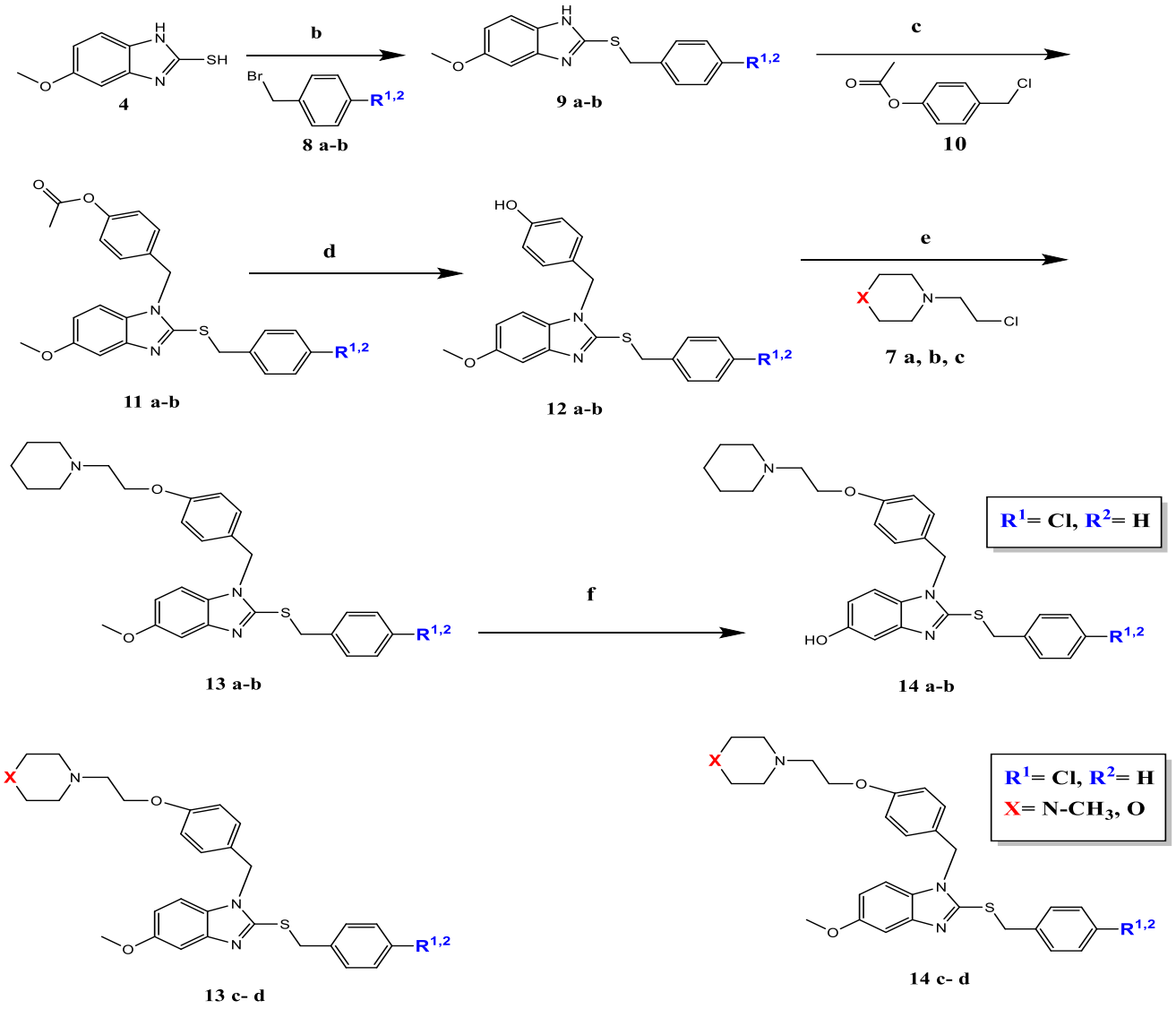
3.2. Synthesis procedure B of 2-(benzylthio)-5-methoxy-1H-benzo[d]imidazole Derivatives (Compound 9 a -b)

A mixture of 5-methoxy 2-mercaptobenzimidazole (1.8 g, 0.01 mole), potassium hydroxide (0.56 g, 0.01 mole) in acetonitrile 50 ml has been heated and stirred under reflux pressure for 15 minutes until the temperature reach 50°C, then 1-bromomethyl benzene derivatives (0.01 mole) was added drop wise, keeping the temperature between (40-50) °C, the reacted mixture was heated and stirred under reflux for 1.30 hours. After cooling, the precipitate filtered and washing with distilled water, crystal was formed by recrystallization from ethanol-water, finally the product weighted and characterization by FT-IR and ^{1}H-NMR [18].

2-((4-chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazole (9a) was obtained as white crystals, 95% yield. IR (FT) λ max cm^{-1}: 3047, 2989, 2972, 2827, 1620, 1593, 628. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ: 12.48 (s, 1H), 7.48 – 6.76 (m, 7H), 4.54 (s, 2H), 3.78 (s, 3H).

2-(benzylthio)-5-methoxy-1H-benzo[d]imidazole (9b) was obtained as off white crystals, 97% yield. IR (FT) λ max cm^{-1}: 3059, 2997, 2951, 2827, 1631.78, 1597, 671. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ: 12.46 (s, 1H), 7.47– 6.75 (m, 7H), 4.54 (s, 2H), 3.78 (s, 3H).





Scheme 1. Synthesis of target compounds 14 a-d. Reagents and conditions; (a) DMF, stirring, K_2CO_3 (b) KOH, acetonitrile, reflux (c) reflux K_2CO_3, KI, reflux (d) NaOH (e) K_2CO_3, DMF, heating (f) $AlCl_3$, ethanthiol.

3.3. Synthesis procedure C of 4-((2-(benzylthio) -5-methoxy-1H-benzo [d]imidazole -1-yl)methyl) phenyl acetate Derivatives (Compound 11a-b)

To stir solutions of intermediate's **9a-b** (0.0315 mole) in acetone (25ml), powder potassium carbonate (0.0347 mole) and potassium iodide (0.0315 mole) were added. The reaction mixture was stirred at room temperature for 10 min. followed by the drop wise addition of 4-chloromethyl phenyl acetate **10** (3.6mmole) solution in *N,N*–dimethylformamide (40 ml). The reaction mixture was then stirred at 80–90°C for 9-12 h., and the reaction progress was monitored using TLC till completion. The workup was initiated by addition of water (25 ml) followed by ethyl acetate (50 ml) and the reaction was allowed to be stirred for an additional 10 min at room temperature. The organic layer was separated, and the aqueous phase was re-extracted with additional ethyl acetate (2 x 25 ml). The organic layers were combined, washed with brine (2 x 25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to obtain the crude products. The final product was purified by recrystallization in ethanol to obtain the corresponding compounds **11a-b** respectively [19].

4-((2-((4-chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazol-1-yl)methyl)phenyl acetate (**11a)** was obtained using the general procedure **C** (80 %). IR (FT) λ max cm^{-1}: 3128, 3062, 2931, 2835, 1759, 1658, 1602. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ 7.86 – 6.71 (m, 11H), 5.35 (s, 2H), 4.76 (s, 2H), 3.78 (s, 3H), 2.64 (s, 3H).

4-((2-((benzyl)thio)-5-methoxy-1H-benzo[d]imidazol-1-yl)methyl)phenyl acetate (**11a)** was obtained using the

general procedure **C** (82 %). IR (FT) λ max cm^{-1}: 2924, 2854, 1759, 1616, 1597.

^{1}H-NMR (400 MHz, DMSO-*d6*) δ 7.41 – 6.69 (m, 11H), 5.46 (s, 2H), 4.45 (s, 2H), 3.69 (s, 3H), 2. 39 (s, 3H).

3.4. Synthesis procedure D of 4-((2-(benzylthio)-5-methoxy-1H-benzo[d]imidazol-1-yl)methyl) phenol Derivatives (Compound 12 a-b)

Sodium hydroxide (6 equivalent, 10% aqueous solution) was added to a solution of intermediate's 11 a- b (1 equivalent) in a 1:1 mixture of methanol and tetrahydrofuran (4 mL/mmol), and the solution was stirred at room temperature for 24 hours. The mixture was poured into dichloromethane (3 mL/mmol) and the organic layer was washed with water (5 mL/mmol). The aqueous layer was acidified to pH=4 with a 1M aqueous hydrochloric acid solution and extracted with dichloromethane (9 mL/mmol). The organic layer was dried over magnesium sulfate, filtered, and the filtrate was concentrated under vacuum and compounds 12a-b were obtained respectively [20].

4-((2-((4-chlorobenzyl)thio)-5-methoxy-1H-benzo[d]imidazol-1-yl)methyl)phenol (**12a)** was obtained using the general procedure **D** (78%) as white powder. IR (FT) λ max cm^{-1}: 3375- 3363, 2935, 1612, 1597. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ 9.61 (s, 1H), 7.50 – 6.64 (m, 11H), 5.48 (s, 2H), 4.61 (s, 2H), 3.77 (s, 3H).

4-((2-((benzyl)thio)-5-methoxy-1H-benzo[d]imidazol-1-yl)methyl)phenol (**12b)** was obtained using the general procedure **D** (76%) as off white powder. IR (FT) λ max cm^{-1}: 3363, 3062, 3024, 2935, 1612, 1597, 698. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ 9.54 (s, 1H), 7.55 – 6.65 (m, 11H), 5.46 (s, 2H), 4.62 (s, 2H), 3.69 (s, 3H).

3.5. Synthesis procedure E of 2-(benzylthio)-5-methoxy-1-(4-(2-(4-methylpiperazin-1-yl)ethoxy) benzyl) -1H-benzo[d]imidazole Derivatives (Compound 13 a-d)

The phenol D1, D2 or D3 (1 eq.), alkyl halide compound A1 or A2 (1.5 eq.), and potassium carbonate (3 eq.) were stirred in N,N-dimethylformamide (4 mL/mmol) and heated to 50°C for 16 hours. The mixture was cooled down to room temperature and poured into water (3 mL/mmol), extracted with ethyl acetate (3 mL/mmol) and the organic layer was then washed with brine (40 mL/mmol). The organic layer was dried over magnesium sulfate, filtered, and the filtrate was evaporated under vacuum and compound E1-4 was obtained respectively [20].

2-((4-chlorobenzyl)thio)-5-methoxy-1-(4-(2-(piperidin-1-yl)ethoxy)benzyl)-1H-benzo[d] imidazole (**13a**) was obtained using the general procedure **E** (78%) as a yellow powder. IR (FT) λ max cm^{-1}: 3047, 2931, 2835, 1662, 1612, 1593, 1091. ^{1}H-NMR (400 MHz, DMSO-d6) δ 7.26 - 6.40 (m, 11H), 4.95 (s, 2H), 4.36 (t, 2H), 4.30 (s, 2H), 3.53 (s, 3H), 2.43(t, 3H), 2.12 (t, 2H), 1.27-1.31(m, 6H).

2-(benzylthio)-5-methoxy-1-(4-(2-(piperidin-1-yl)ethoxy)benzyl)-1H-benzo[d]imidazole(**13b**) was obtained using the general procedure **E** (80%) as a yellow powder. IR (FT) λ max cm^{-1}: 2997, 2935, 2904, 2885, 2835, 1612, 1593, 1107. ^{1}H-NMR (400 MHz, DMSO-d6) δ 7.73 - 6.42 (m, 12H), 5.24 (s, 2H), 4.95 (s, 2H), 4.33 (t, 2H), 3.54 (s, 3H), 2.65 (t, 2H), 2.01 (t, 4H), 1.01-1.31(m, 6H).

2-((4-chlorobenzyl)thio)-5-methoxy-1-(4-(2-(4-methylpiperazin-1-yl)ethoxy)benzyl)-1H benzo[d]imidazole (**13c**) was obtained using the general procedure **E** (74%) as a yellow powder. IR (FT) λ max cm^{-1}: 2997, 2931, 2850, 1612, 1593, 1095. ^{1}H-NMR (400 MHz, DMSO-d6) δ 7.51 - 6.64 (m, 11H), 5.15 (s, 2H), 4.56 (t, 2H), 3.96 (s, 2H), 3.77 (s, 3H), 2.57 (t, 3H), 2.32(t, 2H, 2.75 (m, 8H), 2.90(s, 3H).

4-(2-(4-((2-(benzylthio)-5-methoxy-1H-benzo[d]imidazol-1yl)methyl)phenoxy)ethyl)morpholine (**13d**) was obtained using the general procedure **E** (82%) as a yellow powder. IR (FT) λ max cm^{-1}: 3059, 2935, 2908, 2835, 1620, 1600, 1095. ^{1}H-NMR (400 MHz, DMSO-d6) δ 7.57 - 6.71 (m, 12H), 5.24 (s, 2H), 4.62 (s, 2H), 4.06 (t, 2H), 3.63 (s, 3H), 2.79 (t, 2H), 2.67 (t, 4H), 3.78 (t, 4H).

3.6. Synthesis procedure F of 2-(benzylthio)-5-hydroxy-1-(4-(2-(4-methylpiperazin-1-yl)ethoxy) benzyl) -1H-benzo[d]imidazole Derivatives (Compounds 14 a-d)

30 ml of dichloromethane was added to 0.01 mole of compound 13a-d and 0.035 mole of aluminum chloride. The mixture was agitated at room temperature for 4 hours before (0.025 mole) of ethanethiol was added. After 2.5 hours, 25 ml of dry tetrahydrofuran was added, followed by 5 ml of 20% hydrochloric acid and 25 ml of water. The mixture was stirred overnight and then filtered. The particles were washed with 50 mL of water, followed by 40 mL of diethyl ether, and the result was vacuum dried (compound 14a-d respectively) [21].

2-((4-chlorobenzyl)thio)-1-(4-(2-(piperidin-1-yl)ethoxy)benzyl)-1H-benzo[d]imidazol-5-ol (**14a**) was obtained using the general procedure **F** (55%) as a white powder. IR (FT) λ max cm^{-1}: 3321, 2900, 1612, 1597, 1091. ^{1}H-NMR (400 MHz, DMSO-d6) δ 9.15(s, 1H), 7.23- 6.36 (m, 11H), 4.94 (s, 2H), 4.30 (s, 2H), 4.36 (t, 3H), 2.11(m, 6H), 1.36 – 0.97 (m, 6H). ^{13}CNMR (100 MHz, DMSO-*d6*), ppm: 129.03, 131.24, 132.48, 133.40, 54.87, 150.1, 126.97, 110.82, 118.71, 156.12, 102.10, 55.94, 128.87, 137.30, 137.92, 115.80, 157.41, 67.81, 57.04, 56.08, 56.64, 26.00,

24.49, 141.30; and the ESI-MS for $C_{28}H_{30}ClN_3O_2S$ calculated 507.17; found 508.17 $[M+1]^+$

2-(benzylthio)-1-(4-(2-(piperidin-1-yl)ethoxy)benzyl)-1H-benzo[d]imidazol-5-ol (**14b**) was obtained using the general procedure **F** (71%) as a pale yellow powder. IR (FT) λ max cm^{-1}: 3350- 3059, 3024- 3001, 2935, 1612, 1593, 1103. ^{1}H-NMR (400 MHz, DMSO-d6) δ 7.33- 6.25 (m, 12H), 5.23 (s, 2H), 4.35 (s, 2H), 4.94 (t, 2H), 2.65 (t, 2H), 2.08 (t, 4H), 1.22 – 1.06 (m, 6H). ^{13}CNMR (100 MHz, DMSO-*d6*), ppm: 127.87, 126.86, 126.90, 126.03, 137.08, 36.25, 157.55, 126.03, 111.35, 118.68, 151.09, 101.43, 144.31, 54.68, 128.94, 129.52, 129.61, 115.77, 115.84, 162.77, 56.85, 56.23, 55.93, 56.07, 26.09, 24.57; and the ESI-MS for $C_{28}H_{31}N_3O_2S$ calculated 473.64; found 474.22 $[M+1]^+$

2-((4-chlorobenzyl)thio)-1-(4-(2-(4-methylpiperazin-1-yl)ethoxy)benzyl)-1H-benzo[d]imidazol-5-ol (**14c**) was obtained using the general procedure **F** (70%) as an off-white powder. IR (FT) λ max cm^{-1}: 3387- 3313, 2939, 1620, 1600, 1091. ^{1}H-NMR (400 MHz, DMSO-d6) δ 9.48 (s, 1H), 7.51- 6.63 (m, 11H), 5.50 (s, 2H), 4.61 (s, 2H), 4.55 (t, 2H), 2.29 (t, 2H),), 1.55 (s, 3H), 1.41 – 1.06 (m, 8H). ^{13}CNMR (100 MHz, DMSO-*d6*), ppm: 132.47, 128.88, 129.03, 129.06, 137.24, 35.55, 149.37, 126.99, 110.83, 111.43, 150.80, 101.43, 144.27, 50.01, 127.01, 129.05, 115.39, 115.79, 162.78, 60.02, 56.09, 55.90, 55.95, 46.72; and the ESI-MS for $C_{28}H_{31}ClN_4O_2S$ calculated 475.19; found 476.20$[M+1]^+$

2-(benzylthio)-1-(4-(2-morpholinoethoxy)benzyl)-1H-benzo[d]imidazol-5-ol was obtained using the general procedure **F** (69%) as a white powder. IR (FT) λ max cm^{-1}: 3350, 3008, 2927, 1658, 1612, 1597, 1111. ^{1}H-NMR (400 MHz, DMSO-d6) δ 9.55 (s, 1H), 7.55- 6.58 (m, 12H), 5.22 (s, 2H), 4.62 (s, 2H), 4.07 (t, 3H), 2.79 (t, 2H), 2.11(m, 6H), 2.53 – 2.41 (T, 4H), 3.90 – 3.69 (t, 4H). ^{13}CNMR (100 MHz, DMSO-*d6*), ppm: 127.07, 129.00, 129.11, 127.92, 137.91, 36.73, 162.83, 126.07, 115.07, 111.42, 158.44, 101.49, 144.36, 54.09, 129.59, 129.68, 129.98, 115.81, 115.87, 169.62, 66.65, 57.47, 56.11, 56.26, 65.71; and the ESI-MS for $C_{27}H_{29}N_3O_3S$ calculated 491.20; found 492.20 $[M+1]^+$

4. *In Vitro* cytotoxicity study maintenance of cell cultures

HUVEC (a human normal cell line), SK-OV-3 (a human ovarian cancer cell line) and MDA-MB-231 (a human breast cancer cell line) were purchased from National Cell Bank of Iran (Pasteur Institute, Iran). Cells were grown in RPMI-1640 (Gibco) with 10% FBS (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained at 37 °C under humidified air containing 5% CO_2 and were passaged using trypsin/EDTA (Gibco) and phosphate- buffered saline (PBS) solution. Culturing media and conditions used to grow the cells as 3D colonies was the same as monolayer cell culture [22].

4.1. Cytotoxicity Assay

Cell growth and cell viability were quantified using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide] (Sigma-Aldrich) assay. In brief, for monolayer culture, cells were digested with trypsin, harvested, adjusted to a density of 1.4×10^4 cells/well and seeded to 96-well plates filled with 200 µl fresh medium per well for 24 h. When cells formed a monolayer, they were treated with 100-6.25 µM of the compounds for 24 h at 37 °C in 5% CO_2. At the end of the treatment (24 h), while the monolayer culture was left untouched in the original plate, the supernatant was removed and 200 µl/well of MTT solution (0.5 mg/ml in phosphate-buffered saline [PBS]) was added and the plate was incubated at 37 °C for an additional 4 h. MTT solution (the supernatant of cells was removed and dimethyl sulfoxide was added (100 µl per well). Cells were incubated on a shaker at 37 °C until crystals were completely dissolved [23]. Cell viability were quantified by measuring absorbance at 570 nm using an ELISA reader (Model wave xs2, BioTek, USA). The concentration of the compounds that resulted in 50% of cell death (IC_{50}) was determined from respective dose-response curves [24].

5. Results and Discussion

5.1. *In Vitro* Cytotoxicity Assay

In vitro cytotoxicity of the synthesized compounds (14a-d) are assessed at micro moles (6.25, 12.5, 25, 50, 100 µM) by using the MTT assay. HUVEC (a human normal cell line), SK-OV-3 (a human ovarian cancer cell line) and MDA-MB-231 (a human breast cancer cell line) were employed. The cell proliferation and cytotoxic effect of the synthesized compounds was measured by MTT assay method. The synthesized compounds activity was studied by testing their inhibition ability against normal and cancer cells proliferation. The IC_{50s} were calculated to show how these chemicals inhibited the development of the three cell lines table (1), that were determined from respective dose-response curves as showed in figure 3 [25, 26].

Table 1. IC_{50} values of target compounds 14a- d

Compound	μM		
	HUVEC cell line	MDA-MB-231 cell line	SK-OV-3 cell line
14a	No effect	117.86 ± 0.39	No effect
14b	No effect	574.46± 3.23	125.84± 4.57
14c	55.16± 4.27	24.78± 1.02	71.63± 4.91
14d	No effect	No effect	97.13± 6.77
Raloxifene	37.22	26.73	15.70

As shown in table 1, compound 14c (IC_{50}: 24.78 ±1.02 μM) showed significant reduction in cell viability compared to the other synthesized products against MDA-MB-231 breast cancer cell line. Comparably, lower effects were observed against the human ovarian cancer cell line (SK-OV-3) for all synthesized conjugates 14a-d. In addition, there are no effect of compounds 14a, 14b and 14d against normal cell line HUVEC. Moreover, the activity of all synthesized conjugates 14a-d, were evaluated against raloxifene which showed clear cytotoxic activities with IC_{50} values; 37.22 ± 3.79, 26.73 ± 11.84 and 15.70 ± 1.86 against the tested cell lines HUVEC, MDA-MB-231 and SK-OV-3 with no selectivity worth to mention. From the above-mentioned data (Compound 14c Table 1), there is a clear indication that the synthesized conjugate exert more selective activity against the breast cancer cell line (MDA-MB-231) compared to the other cell line tested. Raloxifene, which is a selective estrogen receptor modulator, is known to bind tightly to estrogen receptor α (ERα), which explains its antiestrogen activity. Raloxifene activity on the molecular level involves a strong interaction with the amino acid Asp-351 in the ligand binding domain of estrogen receptor α (ERα). On the other hand, the nitrogen on piperidine ring side chain of raloxifene shields neutralizes the Asp-351 to produce an antiestrogenic ERα system [27]. Thus, it may clearly be noted that the structure modification of our compounds with piperidine and methyl piperazine side chains is important for its cytotoxic activities. Nevertheless, molecular docking study is needed for the synthesized compounds 14a-d, to confirm the mechanism on the molecular level by they can bind to the alpha estrogen receptors α (ERα) thereby deactivating oncogenic growth pathways in breast cancer cells mediated by estrogen. The molecular docking will be discussed in the next section.

5.2. Molecular docking

For comparison purposes, molecular docking investigations were carried out using Maestro software (Schrödinger, version 2022-1), and ΔG (kcal/mol) as a docking scoring function, compounds **14a-d** and raloxifene were run against Estrogen Receptor Alpha (PDB code: 7KBS). *In vitro* cytotoxicity's results agreed with molecular docking studies, which provided a rationale for the inhibitory activity. Table 2 lists the outcomes of docking with this isoforms, raloxifene was used as a reference to α ER's binding pocket. Figure 4 shows pictures of target substances and raloxifene occupying α ER's binding pocket [28].

Table 2. Docking scores of the designed compounds14a-d, and their interaction with the amino acids residues in the active site of Estrogen Receptor Alpha Ligand Binding Domain (7kbs)

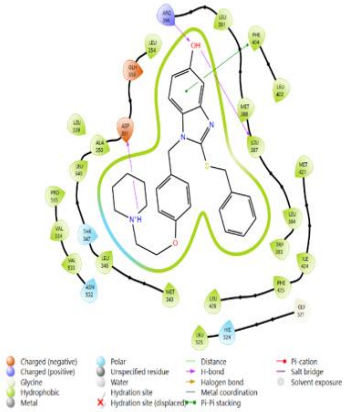
Compound	**Docking score ΔG (kcal/mol)**	***Root Mean Square Deviation (RMSD) score (Å)**	**Amino acids residues involved in the interaction within the active site of Estrogen Receptor Alpha Ligand Binding Domain (7kbs)**
14a	-10.997	0.8053	ASP351, LEU387, PHE404
14b	-11.156	0.6782	ASP351, PHE404, ARG394, LEU387
14c	-9.268	0.8691	ASP351, TRP383, PHE404, ARG394
14d	-8.990	1.0543	ASP351, PHE404, ARG394, H_2O
Raloxifene	-13.454		ASP351, ARG394, PHE404, HIS524

*The RMSD values were calculated from the re-docked raloxifene ligand.

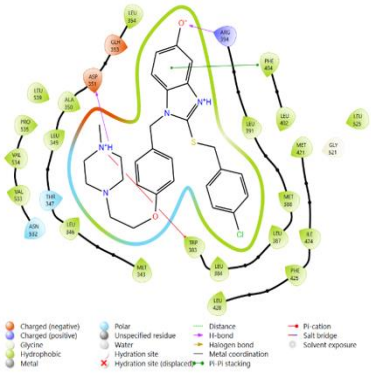
The binding site of estrogen receptor alpha (α-ER) was determined by selecting several residues of amino acids (Table 2). Jordan et al. reported that a strong hydrogen bonding (H-bond) is formed between Asp-351 and the piperidine nitrogen in the wild type α-ER at a distance of 2.7 Å. In his work, it was confirmed that hydrogen bonding between Asp-351 and the nitrogen on piperidine ring of raloxifene, fixes the side chain in raloxifene which in turns shields and neutralizes the negative charge on Asp-351. Their work indicated that amino acid Asp-351 in the ligand binding domain of estrogen receptor α (ERα) is crucial for regulating the estrogen-like activity of selective estrogen receptor modulator ERα complexes [27]. To reveal the inhibitory effects of our newly synthesized compounds

against breast cancer cell line MDA-MB-231, the X-ray derived structure of ER in complex with raloxifene (PDB code: 7KBS) was selected for molecular docking studies. Raloxifene was first docked, and its binding mode and types of interactions were investigated. Our docking results from the best ligand conformation of raloxifene to α ER's binding pocket are shown in figure 4, which revealed hydrogen-bonding interactions between the nitrogen atom of piperidine side chain of raloxifene and the amino acid Asp-351, which is in accordance with the literature. Similar hydrogen bonding interaction can also be observed between the amino acid Arg-394 and the deprotonated phenolic group of benzothiophenes scaffold and the p-hydroxyphenyl with His-524. Furthermore, hydrophobic pi-pi stacking was observed between the benzothiophenes backbone of raloxifene with amino acid Phe-404. The overall docking score of raloxifene to the α ER's showed a value of -13.454 kcal/mol. This model can be used to explain the fitting of our most active derivative 14c within the same binding pocket. Driven from the apparent pharmacophoric commonalities between raloxifene and compound 14c, similar hydrogen bonding between the N-piperazine side chain of the benzimidazole scaffold with the amino acid Asp-351 were found, in addition hydrophobic pi-pi stacking was observed between the benzimidazole core with the amino acid Phe-404. The best docked result of compound 14c is shown in figure 4 with docking score of -9.268 kcal/mol and root mean square deviation (RMSD) of 0.8691 Å compared to the raloxifene X-ray derived conformation (Table 2). Interestingly, the hydrogen bonding observed of raloxifene p-hydroxyphenyl with His-524, is also reported for the ER agonist estradiol [29], is not shown in the binding interactions of the most active compound 14c, which could explain the higher cytotoxic activity of our synthesized compound in comparison to raloxifene against breast cancer cell line MDA-MB-231 (IC_{50}: 24.78± 1.02). In addition to compound 14c, the occupation of the binding of compounds 14a, 14b and 14d along with their docking score; ΔG (kcal/mol) and the RMSD values are shown in table 2, which exposed similar binding affinity towards α ER's binding pocket.

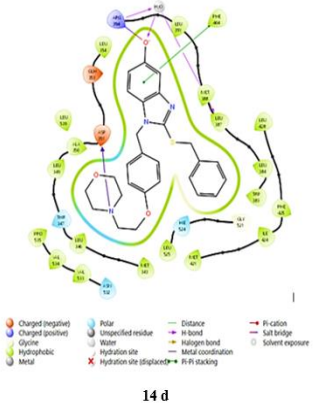
14 a



14 b



14 c



14 d

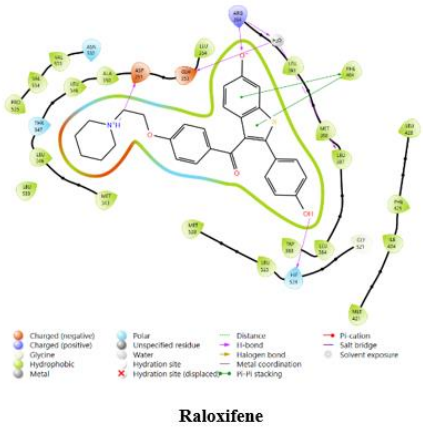


Fig. 4. The occupation of the binding of compounds 14a-d and raloxifene to α-ER

5.3. Chemical Synthesis of intermediates and target compounds 14a-d

The functional groups included into the 2-mercaptobenzimidazole scaffold were selected based on their closeness to the raloxifene structure. The general synthetic pathway for the preparation of the target compounds 14 a-d is outlined in Scheme 1. The preparation of cyclic amine adducts 7a-b, were performed starting form piperidine, methyl piperazine & morpholine with ethylene chlorobromide in a single step using K_2CO_3 as a base catalyst according to literature in good yields. The disappearance of the NH band at 3276 cm^{-1} confirmed the formation of our desired adducts using IR spectroscopy. The first step towards obtaining the desired products 14a-d, starts with the direct S-alkylation of 5-methoxy 2-mercaptobenzimidazole 4, with 1-bromomethyl benzene derivatives (8a-b) using KOH as a catalyst to obtain intermediates 9a-b with excellent yields (95-97%). It was found out that the alteration of 1-bromomethyl benzene derivatives did not play any significant role in the product yields. To obtain the N-alkylated products (12a-b), adducts 9a-b were alkylated with 4-chloromethyl phenyl acetate in good yields, followed by ester hydrolysis using NaOH as base at room temperature. The disappearance of the carbonyl band at 1759 cm^{-1} and the appearance of characteristic phenolic signal between 3100-3300 cm^{-1}, confirmed the formation of inter-mediates 12a-b. The last two steps as outlined in scheme 1, which encompass of O-alkylation of intermediates 12a-b with the previously prepared alkyl chloride derivatives (7a-c) in the presence of K_2CO_3 as a base to give intermediates 13a-d, followed by ether cleavage using ethanethiol/$AlCl_3$ system to furnish the final products (14a-d) successfully in good to moderate yields (55-71%) [20]. During the last two steps (intermediates 13 and 14), the disappearance of the aromatic phenolic signal between 3100-3300 cm^{-1} for the intermediates 13a-d, and reappearance of phenolic signal for the benzimidazole scaffold at 3300 cm^{-1} (14a-d), confirmed the formation of our desired products. Besides, IR spectroscopy, the structure of the synthesized intermediates and final products were confirmed using ^{1}H-NMR, ^{13}C-NMR, and ESI-MS analysis.

6. Conclusion

In the recent decade, there have been several drugs to treat breast and ovarian cancer. However, there is still an unmet need to develop different types of drugs to reduce systemic toxicity and improve therapeutic efficacy. Four new compounds (14 a-d) were synthesized and achieved successfully starting from piperdine, 1-methyl piperazine, morpholine, 5-methoxy-1H-benzo[d] imidazole-2-thiol, 1-bromo-2-chloroethane, 4-chloromethyl phenyl acetate, 1-(bromomethyl)-4-chlorobenzene, 1-(bromomethyl)-4-benzene in acceptable yields and their structures approved by FTIR, ^{1}HNMR, ^{13}CNMR and ESI Mass spectroscopy. They showed cytotoxicity toward MDA-MB-231 and SK-OV-3 cancer cell lines. Their docking analysis and cytotoxicity findings provided a preliminary indication that they are viable [α-ER] candidates.

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