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Research Article / Araştırma Makalesi

MULTI-TARGETED EFFECTS OF NOVEL MELATONIN ANALOGUES: AROMATASE INHIBITION, ER ANTAGONISM AND CYP1 INHIBITORY ACTIVITY

YENİ MELATONİN ANALOGLARININ ÇOKLU HEDEFLİ ETKİLERİ: AROMATAZ İNHİBİSYONU, ER ANTAGONİZMASI VE CYP1 İNHİBİTÖR AKTİVİTESİ

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ABSTRACT

Objective: *This study aimed to synthesize and evaluate novel chlorophenyl-substituted melatonin analogues for their potential multi-target effects in hormone-dependent breast cancer. Their inhibitory activity on aromatase (CYP19A1), antagonistic potential on estrogen receptor (ER), inhibition of CYP1 enzymes, and selective cytotoxicity on cancer cells were investigated.*
Material and Method: *Two groups of novel indole-based melatonin analogues (non-benzylated and benzylated) were synthesized and structurally characterized. Aromatase inhibition was assessed using a cell-free fluorescence-based assay. ER antagonism was evaluated in MCF-7 BUS cells using the E-Screen assay. CYP1 enzyme inhibition was determined via EROD assay using rat liver microsomes. Cytotoxicity was evaluated in MCF-7 BUS and MCF-10A cells using the MTT assay.*
Result and Discussion: *Non-benzylated new compounds (2a–2f) showed higher aromatase and CYP1 inhibition compared to benzylated derivatives (4a–4e). Compound 2f exhibited the highest aromatase (78.5%) and CYP1 (79%) inhibition, and significant ER antagonism. New benzylated compounds displayed stronger cytotoxicity in MCF-7 BUS cells but less enzymatic inhibition. Compound 2f showed a favorable multi-target profile with selective cytotoxicity.*
Keywords: *Aromatase inhibition, breast cancer, CYP1 inhibition, estrogen receptor antagonism, melatonin analogues*

ÖZ

Amaç: *Bu çalışmada, hormon bağımlı meme kanserinde potansiyel çoklu hedefli etkileri araştırılmak üzere klorofenil ikame edilmiş yeni melatonin analoglarının sentezlenmesi ve değerlendirilmesi amaçlanmıştır. Bu bileşiklerin aromataz (CYP19A1) inhibisyonu, östrojen reseptörü (ER) üzerindeki antagonistik potansiyeli, CYP1 enzim inhibisyonu ve kanser hücrelerine karşı seçici sitotoksisiteleri incelenmiştir.*

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Gereç ve Yöntem: *Yeni indol bazlı melatonin analoglarının iki grubu (benzillenmemiş ve benzillenmiş) sentezlenmiş ve yapısal olarak karakterize edilmiştir. Aromataz inhibisyonu hücresiz floresans tabanlı bir test ile değerlendirilmiştir. ER antagonizması, MCF-7 BUS hücrelerinde E-Screen testi ile incelenmiştir. CYP1 enzim inhibisyonu, sıçan karaciğer mikrozomları kullanılarak EROD testi ile belirlenmiştir. Sitotoksisite ise MCF-7 BUS ve MCF-10A hücrelerinde MTT testi ile değerlendirilmiştir.*

Sonuç ve Tartışma: *Yeni benzillenmemiş bileşikler (2a–2f), benzillenmiş türevler (4a–4e) ile karşılaştırıldığında daha yüksek aromataz ve CYP1 inhibisyonu göstermiştir. 2f bileşiği en yüksek aromataz (%78.5) ve CYP1 (%79) inhibisyonunun yanı sıra anlamlı ER antagonizması sergilemiştir. Yeni benzillenmiş bileşikler, MCF-7 BUS hücrelerinde daha güçlü sitotoksisite göstermiş ancak enzimatik inhibisyon düzeyleri daha düşük bulunmuştur. 2f bileşiği, seçici sitotoksisite ile birlikte olumlu bir çoklu hedef profili ortaya koymuştur.*

Anahtar Kelimeler: *Aromataz inhibisyonu, CYP1 inhibisyonu, melatonin analogları, meme kanseri, östrojen reseptör antagonizması*

INTRODUCTION

Breast carcinoma is the most widespread form of cancer affecting women worldwide and is responsible for a considerable proportion of cancer-associated mortality [1]. Despite its higher incidence in developed countries, survival rates are significantly better due to early diagnosis and advanced treatment options. A large proportion of breast cancer cases are hormone-dependent, with estrogen exposure identified as a critical risk factor [2]. Estrogen-induced carcinogenesis is mediated through two primary mechanisms: receptor-mediated cell proliferation and oxidative metabolism, resulting in reactive metabolites that can lead to DNA damage and tumor development [2,3].

In estrogen-driven breast malignancies, two principal therapeutic strategies are employed: agents known as selective modulators of estrogen receptors (SERMs), which block receptor-mediated actions of the hormone, and selective estrogen enzyme modulators (SEEMs), which interfere with the enzymatic pathways responsible for estrogen biosynthesis. Factors such as the various side effects of these drugs and the development of resistance to some drugs during treatment have supported the continued development of new agents that are both preventative against breast cancer and have fewer side effects.

Epidemiological and clinical studies investigating the causes of breast cancer indicate a positive association between exposure to estrogen and breast cancer risk, and that long-term exposure to endogenous estrogens (such as early puberty, late menopause, and nulliparity) are among the factors that increase the risk of breast cancer in humans [4]. Both i*n vitro* and *in vivo* experiments have demonstrated the involvement of two distinct mechanisms in endogenous estrogen-induced carcinogenesis. First one is estrogen receptor-mediated and the second one is estrogen receptor-independent pathways (Figure 1). The first of these is that estrogen binds to the estrogen receptor (ER) and increases cell proliferation. This potential contribution to cancer development is explained by the fact that increasing the number of cell divisions increases the likelihood of errors in DNA replication. Furthermore, rapid cell division may reduce the effectiveness of mutation repair because repair time is insufficient. It has been suggested that in rapidly dividing cells, DNA adducts or breaks can become fixed mutations and be passed on to subsequent generations [5]. Findings from experimental models lacking ERα provide substantial support for the carcinogenic role of estrogen via ER-mediated pathways, and prolonged hormonal exposure has been linked to a higher likelihood of developing breast malignancies [6]. Furthermore, the protective effects of molecules such as tamoxifen and raloxifene, which inhibit estrogen binding to ERα, on breast cancer also provide evidence for ERα-mediated toxicity [7].

Melatonin, a naturally occurring indoleamine secreted by the pineal gland, has shown significant oncostatic effects in hormone-dependent breast cancer [8-12]. Numerous studies suggest that melatonin, secreted primarily from the pineal gland, suppresses the activity of the aromatase enzyme and reduces estrogen biosynthesis [13,14]. The expression of aromatase-related genes is diminished in the presence of melatonin. It achieves this through multiple pathways, including the inhibition of estrogen receptor (ER)-mediated signalling, modulation of local estrogen synthesis, and its potent antioxidant properties [15].

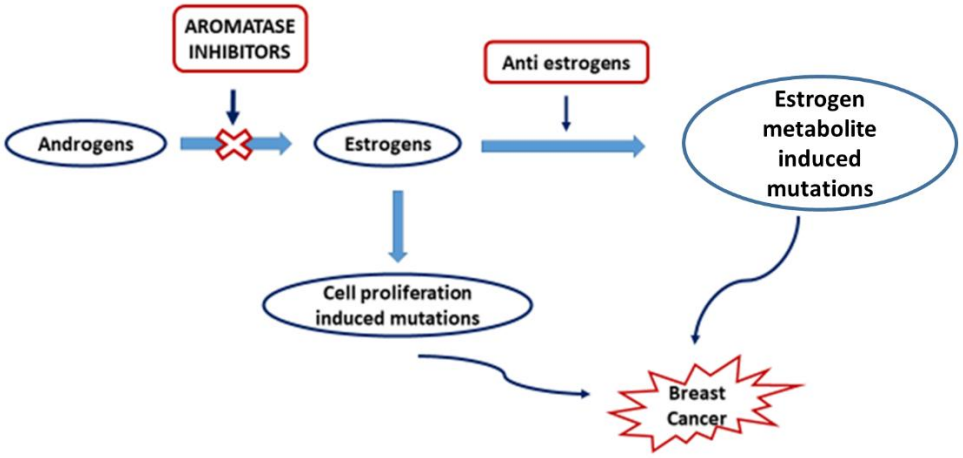


Figure 1. Estrogen receptor-mediated and estrogen receptor-independent pathways in estrogen-induced carcinogenesis

Studies show that an indole derivative melatonin is a particularly good free radical scavenger, and this property, in turn, prevents carcinogenesis. Extensive research indicates that melatonin can suppress estrogen production within breast tissue by inhibiting aromatase; nonetheless, its clinical utility is restricted by certain pharmacokinetic characteristics, including a notably short half-life [2]. Therefore, researchers have turned to the synthesis of new melatonin analogs. Our research group is working on the synthesis/evaluation of the biological activities of several novel indole-based MLT analogs. Through our work with 2-methyl indole derivatives, we determined the aromatase inhibitory activity of these new molecules [16]. We also tested the molecules against cell proliferation in ER+ MCF-7 BUS cells in the media lacking estrogen but supplemented with testosterone. The results showed that halogen-substituted indole derivatives have more potent activity than MLT. This observation was additionally supported through molecular modeling studies. In our next study new 5-fluoroindole derivatives were synthesized and investigated. Within this compound series, three molecules demonstrated antiestrogenic activity in the E-Screen test, displaying IC_{50} values of 3.36×10^{-5} M, 1.31×10^{-7} M, and 1.9×10^{-7} M, respectively. In a direct enzyme-based assay, these compounds were able to reduce aromatase activity by as much as 29% [17]. In our work, we considered it essential to examine how substitutions at various positions on the indole ring influence biological activity in the design of melatonin analogues. Accordingly, we assessed the anticancer and antioxidant properties of a newly synthesized series of indole-7-aldehyde derivatives and further explored their CYP1 inhibitory potential. Our findings revealed that melatonin analogues carrying ortho-halogenated aromatic moieties exhibited pronounced biological activity [18]. In the present work, we synthesized and compared two classes of novel melatonin analogues—5-methoxy-1H-indole derivatives and 1-benzyl-5-methoxy-1H-indole derivatives—to assess their potential as multi-target therapeutic candidates for breast cancer. The designed molecules (Figures 2 and 3) were intended to modulate key molecular pathways implicated in estrogen-driven carcinogenesis. These included aromatase (CYP19A1), a pivotal enzyme in local estrogen biosynthesis; the estrogen receptor (ER), whose activation stimulates proliferation in ER-positive breast cancer cells; and CYP1 family enzymes, which mediate oxidative transformation of estrogen into reactive, potentially carcinogenic metabolites. Furthermore, we evaluated their cytotoxicity profiles in ER-positive MCF-7 and healthy MCF-10A cells via MTT assays, enabling an assessment of selective cytotoxic potential.

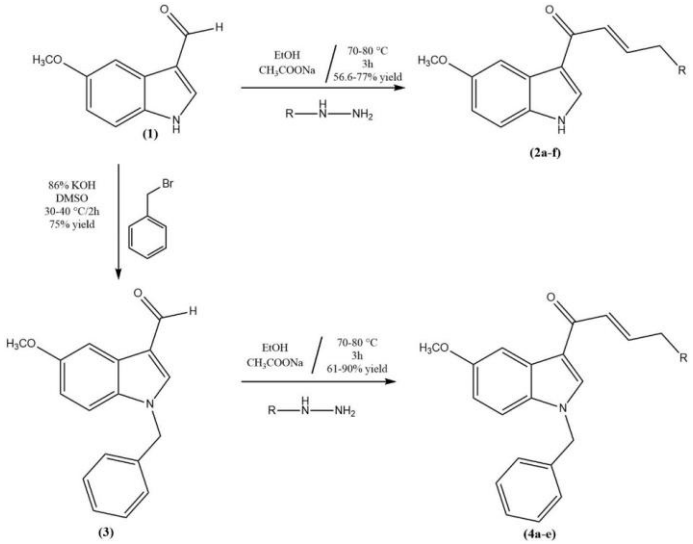


Figure 2. The synthetic pathway of the new indole based melatonin analogues

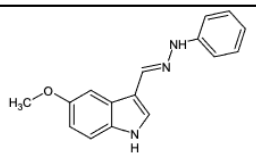
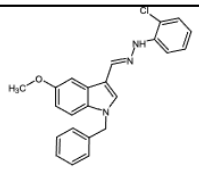
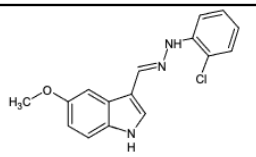
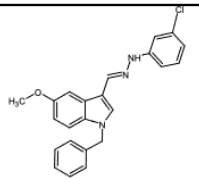
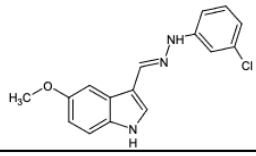
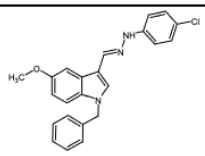
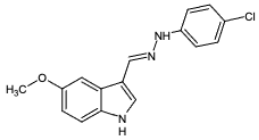
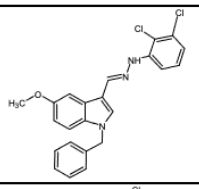
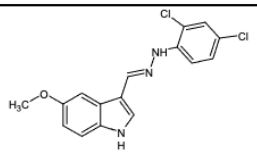
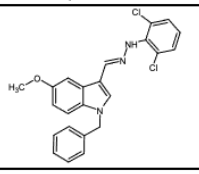
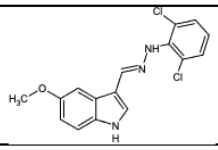
Compound		Compound	
2a		4a	
2b		4b	
2c		4c	
2d		4d	
2e		4e	
2f			

Figure 3. Structures of the compounds

MATERIAL AND METHOD

General Information

MCF-7 BUS cells were generously supplied by Prof. Ana Soto, whereas MCF-10A cells were procured from the American Type Culture Collection (ATCC). The MCF-10A human mammary epithelial line was maintained in DMEM/F12 medium enriched with 5% horse serum, 20 ng/mL human epidermal growth factor, 0.5 µg/ml hydrocortisone, and 10 µg/ml insulin. In contrast, estrogen-responsive MCF-7 BUS breast adenocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Both cell types were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and were passaged upon reaching approximately 80% confluence. Culture media, sera, and additives were obtained from Gibco (Thermo Fisher). Additional reagents, including 17β-estradiol, hydrocortisone, PBS, trichloroacetic acid, and MTT, were sourced from Sigma-Aldrich, while the CYP19/MFC High-Throughput Inhibitor Screening Kit was purchased from BD Gentest. Synthetic chemicals were acquired from Sigma (Germany) and Aldrich (USA). Melting points were determined with a Büchi SMP-20 apparatus; ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Varian 400/100 MHz spectrometer in DMSO-d_6 with TMS as the internal reference; and ESI-MS analyses were performed on a Waters Micromass ZQ system. Rat liver microsomes were kindly provided by Prof. Hilmi Orhan (Department of Pharmaceutical Toxicology, Ege University).

Chemistry

Synthesis of 5-methoxy-1H-indole-3-carbaldehyde (1)

While maintaining the temperature below 10 °C in a water bath, $POCl_3$ was added dropwise to 10 mmol of DMF, and the mixture was stirred magnetically for 30 min. A solution of 10 mmol 5-methoxyindole in 10 ml DMF was then introduced into the reaction mixture. The temperature was gradually increased to 35 °C and stirring continued for 1.5 h, after which the reaction was cooled again to 10 °C. Subsequently, 1 N NaOH was added until the medium reached alkaline pH. The resulting suspension was heated to 60 °C, then allowed to stand until it cooled to ambient temperature. The precipitate formed was collected by filtration and washed thoroughly with water [19]. Compound (1) was obtained in 85% yield.

Synthesis of 1-benzyl-5-methoxy-1H-indole-3-carbaldehyde (3)

Starting from 5-methoxy-1H-indole-3-carboxaldehyde (1) as the starting material, 5-methoxy-N-benzyl-1H-indole-3-carboxaldehyde (3) was synthesized. For this purpose, KOH pellets (86%, 8.6 mmol) were finely ground and gradually added to a previously prepared round-bottom flask containing 22 mL of DMSO. Stirring was carried out for 20 minutes while keeping the temperature between 30 and 35 °C. Subsequently, 10 mmol of compound (1) was added to the reaction mixture, and stirring was continued under reflux at 35–40 °C using a magnetic stirrer for 1.5 hours. At the end of this period, 13 mmol of benzyl bromide was slowly added to the mixture. The mixture was subjected to reflux with magnetic stirring for a further 1.5–2 h. Upon completion, it was diluted with ~50 mL of distilled water and extracted three times using 50 ml portions of ethyl acetate. The combined organic extracts were dried over anhydrous $CaCl_2$, and the solvent was subsequently removed under reduced pressure on a rotary evaporator to afford the crude product. Purification by recrystallization provided the target compound in a 75% yield [20].

General Synthesis of Compounds (2a-f) and (4a-e)

To a solution prepared by dissolving 1 mmol of 5-methoxy-1H-indole-3-carboxaldehyde (1) or 1-benzyl-5-methoxy-1H-indole-3-carbaldehyde (3) in 10 ml of EtOH, 1.3 mmol of phenyl hydrazine HCl or substituted phenyl hydrazine HCl and 4.88 mmol of sodium acetate in distilled water were added. The reaction mixture was stirred in a water bath under reflux at 70-80 °C and the completion of the reaction was determined by monitoring with TLC [21]. The precipitate formed at the end of the reaction was filtered off. If necessary, the resulting crystals were purified by recrystallization from EtOH.

MTT Assay

Cell viability was determined using the MTT assay, which measures the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into purple formazan crystals [22]. Cells were plated at 6×10^3 cells/well and allowed to adhere for 24 h at 37 °C before treatment with 10 µM chlorophenyl derivatives for 72 h. Triton X-100 was included as a cytotoxic control. Following exposure, cultures were rinsed with PBS, incubated with MTT for 4 h, and the resulting formazan was dissolved in DMSO. Absorbance was recorded at 550 nm using a Varioskan Flash plate reader (Thermo Scientific).

Measurement of Aromatase Activity

Aromatase activity was assessed using the CYP19/MFC High-Throughput Screening Kit, which detects the enzyme-mediated conversion of 7-methoxy-4-trifluoromethylcoumarin (MFC) into the fluorescent product hydroxytrifluoromethylcoumarin (HFC) in the presence of a cofactor mix. The procedure followed the protocol reported in our previous work [23]. Aromatase inhibitory effects of compounds were evaluated at 100 µM.

E-Screen Assay

To evaluate antiestrogenic potential, the E-Screen assay-originally introduced by Soto et al. for examining estrogen receptor (ER)-mediated effects [24], was employed. In this assay format, agents that interfere with ER signaling reduce cell proliferation, even when estradiol is present. The method followed previously reported protocols [25] with slight adaptations. In brief, MCF-7 BUS cells were maintained for 144 h in phenol red-free DMEM supplemented with 10% charcoal–dextran-treated FBS, 1% MEM-NEAA, and 1% sodium pyruvate, along with 10^{-10} M estradiol and 1.1 µM chlorophenyl derivatives. Following incubation, cultures were fixed using 10% trichloroacetic acid, stained with 0.4% sulforhodamine B, and their absorbance recorded at 492 nm via a Varioskan Flash plate reader.

Ethoxyresorufin-O-dealkylation (EROD) Assay

CYP1 enzyme activity was evaluated through the O-dealkylation of 7-ethoxyresorufin to its fluorescent product, resorufin, catalyzed by CYP1A1, CYP1A2, and/or CYP1B1 [26]. Inhibitory effects of the chlorophenyl derivatives were tested using phenobarbital-induced rat liver microsomes. Assays were carried out in a 250 µl reaction mixture containing 10 µl microsomes, 90 mM phosphate buffer (pH 7.4), 3.7 µM 7-ethoxyresorufin, 10 µM test compound, and 0.22 mM NADPH. Reactions were incubated at 30 °C for 10 min, terminated with 500 µl acetone. Fluorescence of the supernatants was recorded at 530 nm (excitation) and 586 nm (emission).

Statistical Analysis

All analysis were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), and results are presented as means ± SD. The Shapiro-Wilk test was applied to examine data normality. Statistical significance was evaluated using parametric t-test for data that followed a normal distribution. Data not following normal distribution were analysed via the nonparametric Mann-Whitney test [27]. Differences were considered significant in case of $p<0.05$. Levels of significance are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULT AND DISCUSSION

Melatonin, known for its anticancer properties, has inspired the development of novel derivatives to improve its therapeutic potential. In this study, novel melatonin derivatives were synthesized and evaluated for cytotoxicity, aromatase inhibition, ER antagonism, and CYP1 inhibition, providing insights into their structure-activity relationships and potential in breast cancer therapy.

Structural Characterization of Synthesized Compounds

5-methoxy-3-[(E)-(2-phenylhydrazinylidene)methyl]-1H-indole (2a) Yield: 56.6%, mp: 127-129°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 3H, CH_3), 6.65 (t, 1H, Jo=7.2 Hz, H-4'), 6.82 (dd,

1H, Jo=8.8 Hz, Jm=2.8 Hz, H-6), 7.02 (d, 2H, Jo=8 Hz, H-2',6'), 7.20 (t, 2H, Jo=8 Hz, H-3',5'), 7.30 (d, 1H, Jo=8.4 Hz, H-7), 7.57 (s, 1H, H-2), 7.79 (d, 1H, Jm=2.4 Hz, H-4), 8.09 (s, 1H, hydrazine NH), 9.81 (s, 1H, imine CH), 11.19 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 55.8, 104.2, 111.9, 112.6, 112.9, 113.2, 118.1, 125.4, 128.4, 129.8, 132.7, 135.7, 146.9, 154.7. ESI mass m/z: 266.1 (M+1, %100).

3-{[2-(2-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (2b) Yield: 66.5%, mp: 122-124 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 3H, CH_3), 6.69 (td, 1H, Jo=7.2 Hz, Jm=1.6Hz, H-4'), 6.82 (dd, 1H, Jo=8.8 Hz, Jm=2.4Hz, H-6), 7.23-7.32 (m, 1H, H-3',5',6'), 7.51 (d, 1H, Jo=8 Hz, H-7), 7.59 (s, 1H, H-2), 7.74 (d, 1H, Jm=2.4 Hz, H-4), 8.45 (s, 1H, hydrazine NH), 9.37 (s, 1H, imine CH), 11.41 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 55.8, 104.1, 112.7, 112.8, 113.1, 113.6, 116.3, 118.9, 125.4, 128.7, 129.3, 129.9, 132.7, 139.5, 142.8, 154.8. ESI mass m/z 300 (M+1, %100), 302 (M+1+2, %34).

3-{[2-(3-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (2c) Yield: 70%, mp: 100-102 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 3H, CH_3), 6.68 (dd, 1H, Jo=8 Hz, Jm=1.6 Hz H-6'), 6.83 (dd, 1H, Jo=8.8 Hz, Jm=2.8 Hz, H-6), 6.88 (d, 1H, Jo=7.2 Hz, H-4'), 7.12-7.13 (m, 1H, H-2'), 7.21 (t, 1H, Jo=8.4Hz, H-5'), 7.32 (d, 1H, Jo=8.8 Hz, H-7), 7.62 (d, 1H, Jm=2.8 Hz, H-2), 7.77 (d, 1H, Jm=2 Hz, H-4), 8.11 (s, 1H, hydrazine NH), 10.09 (s, 1H, imine CH), 11.24 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6,100 MHz): 54.8, 103.0, 109.9, 110.4, 112.1, 112.2, 112.3, 116.6, 124.5, 128.2, 130.6, 131.8, 133.8, 136.2, 147.5, 154. ESI mass m/z 300.2 (M+1, %100).

3-{[2-(4-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (2d) Yield: 64.5%, mp: 188-189°C; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 3H, CH_3), 6.80 (dd, 1H, Jo=8.8 Hz, Jm=2.8 Hz, H-6), 6.99 (d, 2H, Jo=9.2 Hz, H-2',6'), 7.21 (d, 2H, Jo=8.8 Hz, H-3',5'), 7.29 (d, 1H, Jo=8.8 Hz, H-7), 7.58 (s, 1H, H-2), 7.72 (d, 1H, Jm=2.4 Hz, H-4), 8.08 (s, 1H, hydrazine NH), 9.95 (s, 1H, imine CH), 11.21 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 55.8, 104.1, 112.7, 112.9, 113.0, 113.3, 121.2, 125.3, 128.8, 129.6, 132.6, 136.6, 145.8, 154.8. ESI mass m/z 300 (M+1, %100).

3-{[2-(2,4-dichlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (2e) Yield: 61%, mp: 155-156 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 3H, CH_3), 6.82 (dd, 1H, Jo=8.8 Hz, Jm=2.4 Hz, H-6), 7.29-7.41 (m, 2H, H-5',6'), 7.48 (d, 1H, Jo=8.8 Hz, H-7), 7.51 (d, 1H, Jm=2.4 Hz, H-3'), 7.69 (d, 1H, Jm=2.4 Hz, H-4), 7.91 (s, 1H, H-2), 8.46 (s, 1H, hydrazine NH), 9.54 (s, 1H, imine CH), 11.32 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 21.1, 76.7, 77.0, 77.3, 112.56, 114.9, 121.8, 123.4, 124.9, 129.2, 135.1. ESI mass m/z 335 (M+1, %100), 337 (M+1+2, % 63), 339 (M+1+4, %14).

3-{(E)-[2-(2,6-dichlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (2f) Yield: 66%, mp: 168-169 °C; ^{1}H-NMR (DMSO-d_6 400 MHz): δ 3.75 (s, 3H, CH_3), 6.80 (dd, 1H, Jo=8 Hz, Jm=2 Hz, H-6), 6.96 (t, 1H, H-4'), 7.3 (d, 1H, JO=8.8 Hz, H-7), 7.43 (d, 2H, Jo=8 Hz, H-3',5'), 7.58 (s, 1H, H-2), 7.77 (s, 1H, H-4), 8.35 (s, 1H, hydrazine NH), 8.84 (s, 1H, imine CH), 11.24 (s, 1H, indole NH). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 55.1, 103.7, 112, 112.2, 112.3, 122.6, 124.6, 124.7, 128.4, 129.3, 131.9 138.8 (azometin C), 139.1, 154.1. ESI mass m/z 334 (M+1, %100), 336 (M+1+2, % 63), 338 (M+1+4, %14).

1-benzyl-3-{(E)-[2-(2-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (4a) Yield: 77%, mp: 128-130 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 3.98 (s, 3H, CH_3), 5.32 (s, 2H, CH2), 6.79 (td, 1H, JO=6 Hz, Jm=1.2 Hz, H-4'), 6.95 (dd, 1H, Jo=7.12 Hz, Jm=2 Hz, H-6''), 7.15-7.67 (m, 9H, Ar-H), 7,66 (dd, 1H, Jo=6.56 Hz, Jm=1.6 Hz, H-6), 7.83 (s, 1H, hydrazone-NH), 7.97 (d, 1H, Jm=2 Hz, H-4) 8.10 (s, 1H, azomethine-CH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 50.53, 55.76, 104.24, 110.72, 112.18, 113.36, 113.60, 116.65, 119.07, 125.99, 126.83, 127.90, 128.90, 129.06, 130.24, 132.54, 136.76, 141.37, 155.26 (azomethine-C). ESI mass m/z: 390 (M+1, %100), 392 (M+1+2, %30).

1-benzyl-3-{(E)-[2-(3-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (4b) Yield: 90%, mp: 153-155 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 3.99 (s, 3H, CH_3), 5.27 (s, 2H, CH2), 6.790 (d, 1H, JO=6 Hz, H-6), 6.855 (d, 1H, JO=8 Hz, H-7), 6.92 (dd, 1H, Jo=8.8 Hz, Jm=2.4 Hz, H-6''), 7.11-7.34 (m, 9H, Ar-H), 7.40 (s, 1H, hydrazone-NH), 7.93 (s, 1H, azomethine-CH) 7.96 (d, 1H, Jm=2.4 Hz, H-4). ^{13}C-NMR (DMSO-d_6, 100 MHz): 50.46, 55.63, 103.67, 110.43, 110.75, 112.19, 112.45, 113.72, 118.91, 125.83, 126.81, 126.96, 127.88, 128.62, 128.88, 129.89, 130.17, 132.39, 135.18, 135.27, 136.81, 146.64, 155.26 (azomethine-C). ESI mass m/z 390 (M+1, %100), 392 (M+1+2, %34)

1-benzyl-3-{(E)-[2-(4-chlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (4c) Yield:

61%, mp: 159-160 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 3.96 (s, 3H, CH_3), 5.29 (s, 2H, CH2), 6.94 (dd, 1H, Jo=7.08 Hz, Jm=2 Hz, H-6), 7.06-7.35 (m, 12H, Ar-H), 7.9363 (d, 1H, Jm=2 Hz, H-4), 7.96 (s, 1H, azomethine-CH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 50.48, 55.74, 104.16, 110.69, 112.22, 113.35, 113.46, 125.92, 126.82, 127.89, 128.88, 129.89, 132.25, 135.20, 136.80, 155.22 (azomethine-C). ESI mass m/z 390 (M+1, %100), 392 (M+1+2, %34)

1-benzyl-3-{(E)-[2-(2,3-dichlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (4d) Yield: 76%, mp: 142-144 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 3.45 (s, 3H, CH_3), 5.28 (s, 2H, CH_2), 6.91-6.94 (m, 2H, H-6 ve H-7), 7.12-7.34 (m, 8H, Ar-H), 7.543 (dd, 1H, Jo=8.4 Hz, Jm=1.2 Hz, H-6''), 7.88 (s, 1H, hydrazone-NH), 7.96 (d, 1H, Jm=2.4 Hz, H-4). 7.91 (d, 1H, Jm=2.4 Hz, H-4), 8.07 (s, 1H, azomethine-CH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 50.58, 55.77, 104.19, 110.85, 111.67, 112.61, 113.34, 114.82, 119.60, 125.98, 126.83, 127.17, 128.78, 128.90, 129.12, 130.97, 131.32, 132.51, 132.64, 136.57, 138.07, 142.84, 155.36 (azomethine-C). ESI mass m/z: 424 (M+1, %100), 426 (M+1+2, % 73), 338 (M+1+4, %22)

1-benzyl-3-{(E)-[2-(2,6-dichlorophenyl)hydrazinylidene]methyl}-5-methoxy-1H-indole (4e) Yield: 62%, mp: 172-174 °C; ^{1}H-NMR (DMSO-d_6, 400 MHz): 4.00 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 6.80 (s, 1H, H-2), 6.94 (dd, 1H, Jo=7.08 Hz, Jm=2 Hz, H-6), 7.03 (s, 2H, H-2'' ve H-6''), 7.13-7.35 (m, 7H, Ar-H), 7.46 (s, 1H, hydrazone-NH), 7.93 (d, 1H, Jm=1.92 Hz, H-4), 7.96 (s, 1H, azomethine-CH). ^{13}C-NMR (DMSO-d_6, 100 MHz): 50.53, 55.56, 103.41. 110.71, 110.87, 111.93, 113.98, 118.64, 125.78, 126.83, 127.93, 128.91, 130.21, 132.38, 135.51, 136.18, 136.70, 147.09, 155.40 (azomethine-C).ESI mass m/z 424 (M+1, %100), 426 (M+1+2, % 63), 338 (M+1+4, %17)

Aromatase Inhibitory Effects

The aromatase inhibitory potential of chlorophenyl derivatives was evaluated at a concentration of 100 µM using a fluorescent substrate (MFC) in an *in vitro* cell-free assay. Aromatase activity was reduced to 25.4%, 25%, 47%, 31%, 21%, and 21.5% by compounds 2a-2f, respectively, demonstrating significant inhibition across the series. In contrast, the activity was reduced to 85%, 67%, 54%, 81%, and 80% by benzyl-substituted compounds 4a-4e, respectively. A comparative analysis between the two groups revealed that the aromatase inhibition potential significantly decreased with the introduction of benzyl substitutions (Figure 4). Compounds in the non-substituted group (2a-2e) demonstrated significant inhibition of aromatase activity, with reductions in enzyme activity ranging from 43% to 89%. Among the synthesized compounds, those with halogen substitutions, particularly dichlorophenyl groups, exhibited enhanced aromatase inhibition. This aligns with prior studies showing that halogenation increases the binding affinity of compounds to the active site of cytochrome P450 enzyme [16,25], and is further supported by recent reports demonstrating that enantioenriched *β*-indolyl ketones bearing halogenated aromatic substituents exhibit enhanced aromatase inhibitory activity through stronger heme–ligand interactions within the CYP19A1 active site [28]. In contrast, the benzyl-substituted derivatives (4a-4e) showed a markedly lower inhibition of aromatase activity, with reductions ranging from 15% to 33%. This decline suggests that the bulkier benzyl group may hinder proper binding to the enzyme's active site, potentially due to steric clashes or altered electronic interactions. These observations are consistent with prior studies indicating that larger substituents on melatonin analogues can reduce their effectiveness as aromatase inhibitors.

Estrogen Receptor Antagonist Effects

The ER antagonistic effects of the chlorophenyl derivatives were evaluated using the E-Screen assay. To determine the antiestrogenic potential of the compounds, MCF-7BUS cells were treated with 0.1 nM estradiol and 1.1 µM of each compound, and the proliferative effect (PE) was calculated. All tested derivatives significantly inhibited cell proliferation even in the presence of estradiol. Notably, PE values of 1 or below were observed for 2c, 2f, and all benzyl-substituted compounds (4a-4e), suggesting that these compounds may act as potent estrogen receptor antagonists (Figure 5).

Compounds 2a, 2f, and 4c, which exhibited PE values ≤1, demonstrated strong ER antagonistic activity, aligning with their potential application in estrogen receptor-positive (ER+) breast cancer therapy. The dichlorophenyl substitution appears to be a critical determinant for this activity, as seen in the case of 2f. This observation is in agreement with previous reports demonstrating that halogenated

phenyl groups can enhance ER antagonistic activity [17,25].

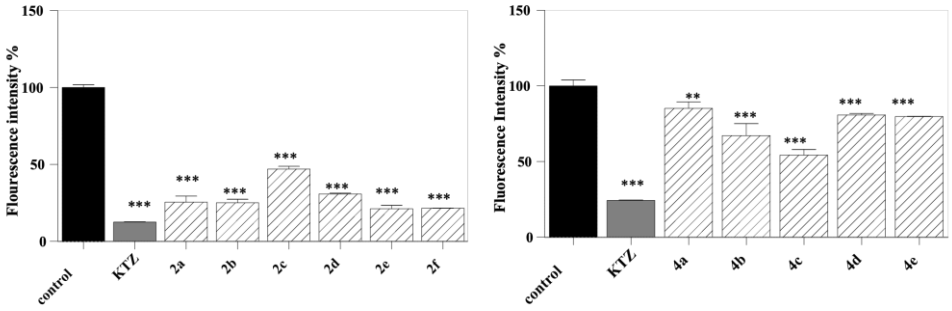


Figure 4. Effects of compounds on aromatase activity at 100 µM. Bars represent percentage values compared to control group from three different experiments (n:3,N:3). KTZ: Ketoconazole. *p<0.05, **p<0.01, ***p<0.001

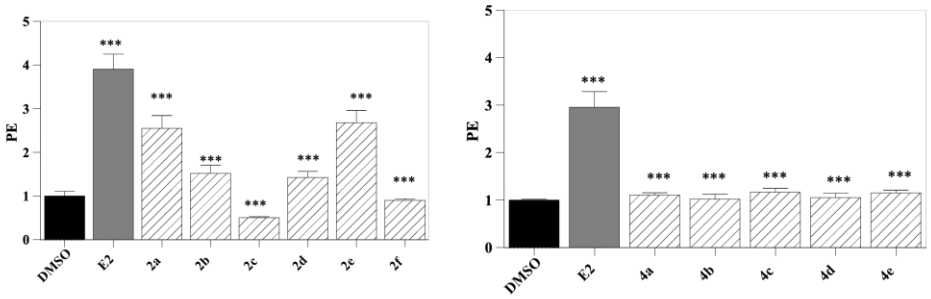


Figure 5. Antiestrogenic effects of compounds (1.1µM) in MCF-7 BUS cells. Statistical analyses were performed by comparing samples with E2 for antiestrogenic activity. Bars represent 'medium ± standard deviation' values from three different experiments (n:3,N:3). PE: proliferative effect E2:Estradiol DMSO: Dimethylsulfoxide *p<0.05, **p<0.01, ***p<0.00

CYP1 Inhibitory Effects

The CYP1 inhibitory potential of chlorophenyl derivatives was evaluated using microsomes and 7-ethoxyresorufin as a substrate. Ketoconazole, used as a positive control, inhibited CYP1 activity by 55%. Compounds 2a-2f demonstrated higher inhibitory effects compared to ketoconazole, with inhibition rates of 67%, 54%, 76%, 64%, 62%, and 79%, respectively. The benzyl-substituted derivatives (4a-4e) also significantly inhibited CYP1 activity, with inhibition rates of 37%, 43%, 42%, 26%, and 23%, respectively. However, their inhibitory effects were notably lower than those of the non-substituted group, indicating that the presence of benzyl substitution reduces CYP1 inhibition potential (Figure 6). These results align with prior studies showing that compact and electron-rich structures enhance binding to the active site of CYP1 enzymes [29,30].

The benzyl-substituted derivatives (4a-4f) exhibited relatively low activity in both aromatase inhibition and CYP1 enzyme inhibition assays. This trend suggests that the bulky benzyl group impairs the interaction of these compounds with the active sites of both enzymes, likely due to steric hindrance and altered electronic interactions.

Cell Viability

To assess the cytotoxicity of novel chlorophenyl derivatives on non-tumorigenic (MCF-10A) and breast cancer MCF-7 BUS cell lines, using the MTT assay. Among the tested compounds, MCF-10A cell viability decreased to 74% with 2a and 2d, 81% with 2f, and 72% with Y9. Notably, within the 2a-2f series, only 2f significantly reduced MCF-7 BUS cell viability to 57%. In contrast, all

benzyl-substituted analogues (4a–4e) produced pronounced cytotoxic effects in MCF-7 BUS cells, indicating greater potency against the cancer cell line (Figure 7).

Among the synthesized compounds, those with benzyl substitutions (4a-4e) demonstrated enhanced cytotoxic effects in MCF-7 cells compared to the non-substituted group (2a-2f), suggesting that the addition of benzyl groups may increase membrane permeability and receptor-binding affinity [31]. However, their aromatase inhibition potential was notably lower than that of non-benzylated derivatives. This indicates that while benzyl groups contribute to cytotoxicity, they might sterically hinder the interaction with the active site of the aromatase enzyme [32]. Compound 2f, a non-benzylated derivative, stood out with superior inhibition of aromatase activity and a notable reduction in MCF-7 BUS cell viability, making it a promising lead compound for further optimization.

The cytotoxicity assays revealed selective activity of the compounds against cancer cells, with minimal impact on MCF-10A cells. This selective cytotoxicity suggests that these compounds could effectively target cancerous tissues while sparing normal cells, a crucial attribute for potential therapeutic agents. However, the benzylated derivatives, while potent against MCF-7 cells, showed moderate effects on MCF-10A, indicating the need for further structural refinement to improve selectivity.

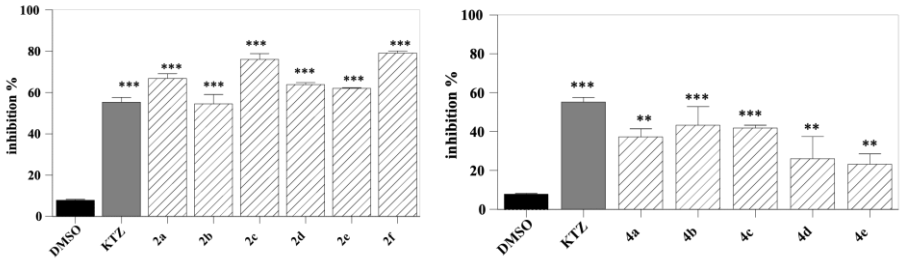


Figure 6. Microsomal CYP1 inhibitory effects of compounds (10µM). Bars show percentage inhibition values compared to control group from three different experiments (n:3,N:3). KTZ: Ketoconazole. *p<0.05, **p<0.01, ***p<0.001

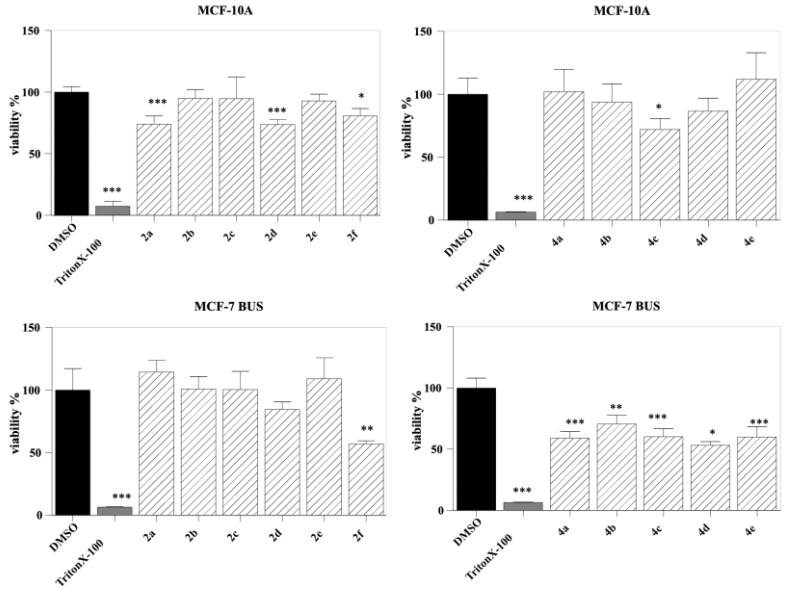


Figure 7. Effects of compounds on MCF-10A and MCF-7 BUS cell viability at 10 µM, as determined by the MTT assay. Bars represent the percentage of viable cells relative to the DMSO-treated control group, based on three independent experiments(n:3,N:3) . DMSO: Dimethyl sulfoxide. Statistical significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001

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AUTHOR CONTRIBUTIONS

Concept: E.I.E., H.G.O.; Design: E.I.E., S.S., H.G.O.; Control: S.S., H.G.O.; Sources: S.S., H.G.O.; Materials: S.S., H.G.O.; Data Collection and/or Processing: E.I.E., H.Ş., B.E.; Analysis and/or Interpretation: E.I.E., H.Ş., B.E.; Literature Review: E.I.E., H.Ş., B.E., S.S., H.G.O.; Manuscript Writing: E.I.E., B.E., S.S.; Critical Review: E.I.E., H.Ş., B.E., S.S., H.G.O.; Other: -

CONFLICT OF INTEREST

The authors declare there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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