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Research Article

Investigation of the Effects of Benzimidazole Derivatives on the mTOR Pathway in Breast Cancer

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Abstract

The mammalian target of rapamycin (mTOR) pathway plays a critical role in cancer progression, making it a key target for therapeutic intervention. Dysregulation of mTOR signaling is frequently observed in malignancies, highlighting the need for potent and selective inhibitors. In this study, a series of benzimidazole derivatives were designed and evaluated for their potential as mTOR inhibitors. Cytotoxicity assessments using MTT assays demonstrated that compounds 10 and 15 exhibited significant anti-proliferative effects against breast cancer cell lines, with IC_{50} values of 6.63 µM and 5.28 µM, respectively. Further biochemical studies revealed that the most active compounds effectively suppressed mTOR phosphorylation at Ser2448 in MCF-7 cells, as confirmed by colorimetric enzymatic activity assays. These results suggest that compound 15, in particular, represents a promising lead for the development of novel mTOR-targeted therapies. This study provides valuable insights into the structure-based design of mTOR inhibitors, offering a foundation for future advancements in targeted cancer treatment.
Keywords: Benzimidazole, mTOR inhibitory activity, structure-activity relationship

Benzimidazol Türevlerinin mTOR Yolağı Üzerindeki Etkilerinin Meme Kanserinde Araştırılması

Öz

Rapamisin memeli hedefi (mTOR) sinyal yolu, kanser ilerlemesinde kritik bir rol oynayarak terapötik müdahaleler için önemli bir hedef haline gelmiştir. mTOR sinyal iletiminin düzensizliği, malignitelerde sıkça gözlemlenmekte olup, güçlü ve seçici inhibitörlere olan ihtiyacı vurgulamaktadır. Bu çalışmada, bir dizi benzimidazol türevi tasarlanmış ve potansiyel mTOR inhibitörleri olarak değerlendirilmiştir. MTT analizleri ile yapılan sitotoksisite değerlendirmeleri, 10 ve 15 numaralı bileşiklerin meme kanseri hücre hatlarına karşı belirgin anti-proliferatif etki gösterdiğini ortaya koymuş ve IC_{50} değerleri sırasıyla 6,63 µM ve 5,28 µM olarak belirlenmiştir. Biyokimyasal incelemeler, en aktif bileşiklerin MCF-7 hücrelerinde Ser2448 bölgesinde mTOR fosforilasyonunu etkili bir şekilde baskıladığını ve bu durumun kolorimetrik enzim aktivite testleriyle doğrulandığını göstermiştir. Elde edilen bulgular, özellikle 15 numaralı bileşiğin yeni mTOR hedefli tedavilerin geliştirilmesi için umut verici bir öncü olabileceğini ortaya koymaktadır. Bu çalışma, mTOR inhibitörlerinin yapı bazlı tasarımına dair değerli bilgiler sunarak hedefe yönelik kanser tedavilerinin gelecekteki gelişmelere katkı sağlayacaktır.
Anahtar Kelimeler: Benzimidazol, mTOR inhibitör aktivite, yapı-aktivite ilişkisi

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1. Introduction

The mammalian target of rapamycin (mTOR) signaling pathway plays a crucial role in regulating cellular processes such as growth, metabolism, and survival, making it a critical target in cancer research. Dysregulated mTOR activity, often resulting from mutations in upstream regulators such as PTEN and PIK3CA, is implicated in the pathogenesis of multiple cancers, including breast, lung, and prostate malignancies. This aberrant signaling promotes tumor proliferation, enhances metastatic potential, and contributes to resistance against conventional therapies. Consequently, mTOR inhibition has emerged as a promising therapeutic strategy in precision oncology [1].

As a central regulator, mTOR integrates extracellular and intracellular cues, including growth factors (e.g., IGF-1, VEGF), nutrient availability, and energy status, predominantly activating through the PI3K-Akt pathway. Tumor suppressors such as PTEN and the tuberous sclerosis complex (TSC1/2) serve as key negative regulators, preventing excessive mTOR activation. The mTOR protein functions within two distinct complexes: mTORC1, which governs protein synthesis and cell growth, and mTORC2, which regulates cytoskeletal dynamics and Akt signaling. Given the critical involvement of these complexes in oncogenesis, targeted inhibition of mTOR has been widely explored for cancer treatment [2].

The discovery of rapamycin and its analogs (rapalogs), such as everolimus and temsirolimus, provided the first generation of mTOR inhibitors, primarily targeting mTORC1. However, feedback activation of upstream signaling pathways limited their therapeutic efficacy in certain cancers [3]. To overcome these limitations, next-generation mTOR inhibitors, including pan-mTOR inhibitors like torkinib and INK128, were developed to simultaneously target both mTORC1 and mTORC2, exhibiting improved antitumor activity. Additionally, combining mTOR inhibitors with agents targeting parallel pathways, such as PI3K or MEK inhibitors, has shown promise in overcoming resistance mechanisms [4].

Benzimidazole, a privileged scaffold in medicinal chemistry, has gained attention for its anticancer properties, particularly in the design of PI3K and mTOR inhibitors. The benzimidazole core is frequently incorporated into small-molecule inhibitors targeting PI3K/mTOR signaling, with several derivatives exhibiting potent anticancer activity in both *in vitro* and *in vivo* models [5, 6]. The benzimidazole scaffold is commonly found in PI3K inhibitors, such as the pan-PI3K inhibitor ZSTK474 (Fig. 1A), selective PI3K inhibitors (Fig. 1B), and dual PI3K/mTOR inhibitors (Fig. 1C) [7]. These compounds demonstrate excellent druglikeness and exhibit significant antitumor effects both *in vitro* and *in vivo* [8, 9].

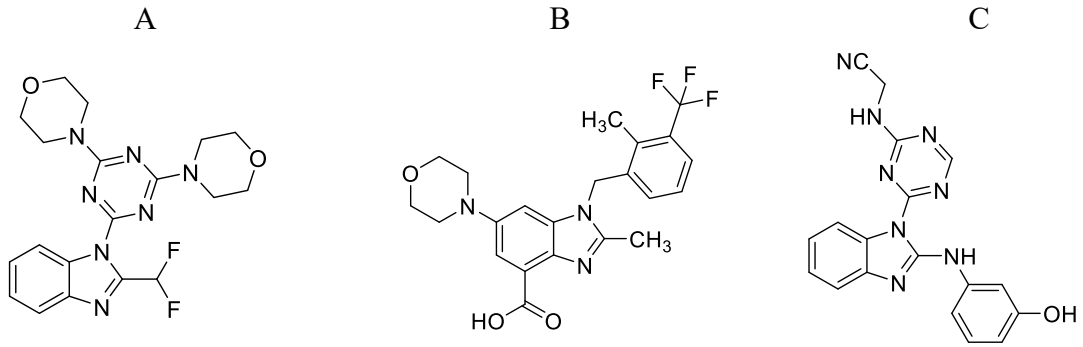
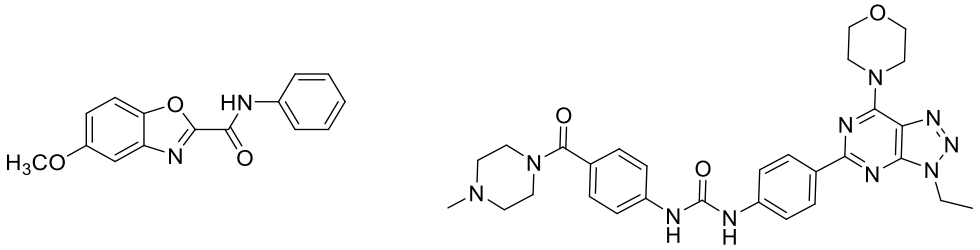


Fig. 1. The structures of benzimidazole-based pan-PI3K, PI3K, and dual PI3K/mTOR inhibitors

In addition, bioisosteric modifications, such as benzoxazole-containing analogs (e.g., RBK-13), have demonstrated significant mTOR inhibition, leading to apoptosis and cell cycle arrest in cancer cell lines [10]. Compounds like PKI-402, which simultaneously inhibit PI3K and mTOR, have been shown to suppress tumor proliferation and overcome therapeutic resistance (Fig. 2) [11].



RBK-13 **PKI-402**

Fig. 2. Some mTOR Inhibitor Compounds

Despite these advancements, challenges remain in optimizing selectivity and efficacy in mTOR-targeted therapies. The rational design of dual or multi-target inhibitors, along with the integration of novel drug delivery strategies, holds great potential for improving treatment outcomes [12].

In this study, we designed and synthesized a series of 2-phenyl benzimidazole derivatives, incorporating a methyl group as a substituent at the 4-position of the benzimidazole ring to enhance mTOR inhibitory activity. These compounds were evaluated for their potential as breast cancer proliferation inhibitors, with the goal of advancing new therapeutic strategies in oncology.

2. Material and Methods

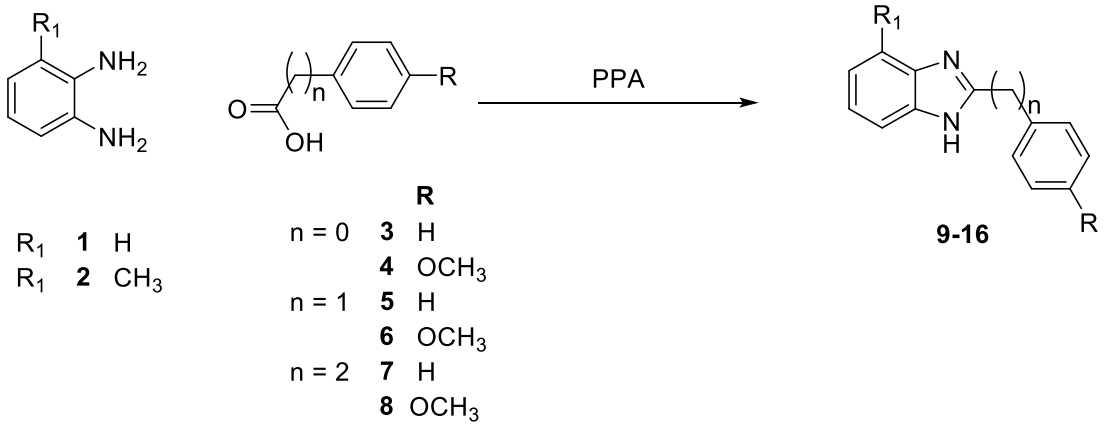
2.1. Chemistry

The reagents and solvents used were obtained from Sigma–Aldrich chemicals. Reagents and solvents are used directly without further purification. Reaction progress and product mixtures were monitored by thin layer chromatography (TLC) using E-Merck 0.25 silica gel plates. Colorless products were detected by UV light (254 nm). Melting points were determined by using one end open capillary tubes on an uncorrected Analab melting point apparatus. ^{1}H- and

^{13}C-NMR spectra were recorded on Bruker Avance 400 spec-trometers, operated at 400 MHz for 1H and 100 MHz for ^{13}C nuclei, relative to TMS as the internal standard on the δ (ppm) scale with deuterated dimethylsulfoxide (DMSO-d_6) and chloroform ($CDCl_3$) as the solvents. The IR spectra were obtained on a Perkin Elmer Spectrum One FT-IR spectrometer.

2.1.1. General procedure for the synthesis of non- or disubstituted benzimidazole derivatives (9-16)

1,2-phenylenediamine derivative (**1** and **2**) (1 eq.) and corresponding carboxylic acid derivative (**3-8**) (1,1 eq.) were refluxed under a reflux condenser with a magnetic stirrer for a period of 13-15 hours after being dissolved in polyphosphoric acid (PPA) and heated in an oil-bath at 150 °C. The reactions were followed by TLC. After cooling, the reaction mixture was poured onto ice water and neutralized by mixing with 5N NaOH until being of slightly basic pH (8–9) to get the precipitate. The resulting precipitate was filtered off and washed with cold water. Then compounds purified by flash column chromatography finally crystallized with a suitable solvent. The resulting crystalline compounds were filtered, and the vacuumed product was dried (Scheme 1).



Scheme 1. General synthesis method of the targeted compounds.

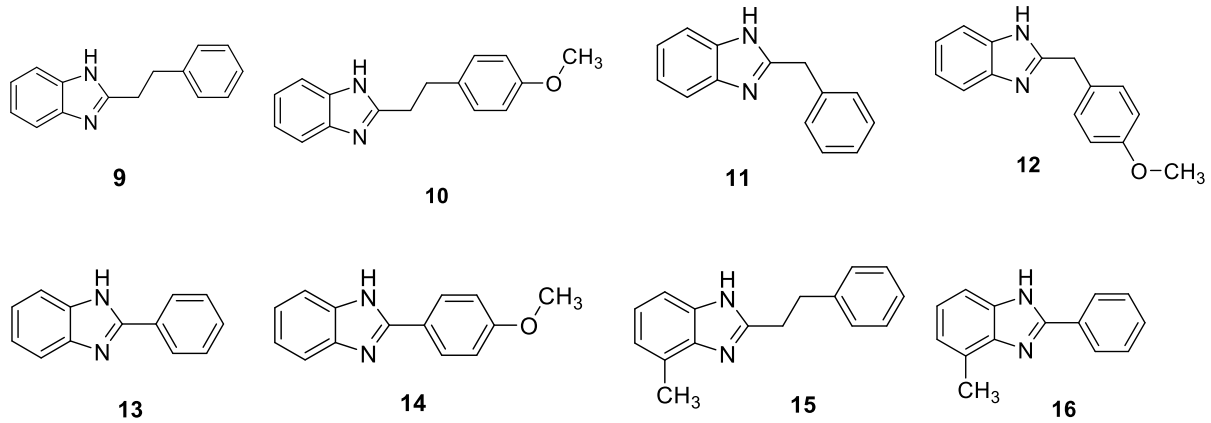


Fig. 3. Structures of the targeted benzimidazole derivatives.

The synthesis of the benzimidazole derivatives (fig. 3) was performed according to the procedure described in our previous studies [13-17]. The NMR spectra are in agreement with the reported data.

*2-Phenethyl-1H-benzo[d]imidazole (**9**)* [19]
Yield: 50%; White solid; mp: 199–202 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.26 (dd, 2H, J = 7.3, 8.3 Hz, -CH_2), 3.51 (dd, 2H, J = 7.3, 8.3 Hz, -CH_2), 7.30–7.35 (m, 5H, Ar–H), 7.47–7.53 (m, 2H, Ar-H), 7.75–7.80 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 150.2, 141.6, 131.9, 129.4, 128.9, 128.5, 127.1, 122.8, 120.5, 115.2, 110.8, 35.2, 33.4.

*2-(4-Methoxyphenethyl)-1H-benzo[d]imidazole (**10**)* [18]
Yield: 55%; White solid; mp: 195–196 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.53 (br s, 1H, NH), 7.23 (dd, J = 3.17, 6.10 Hz, 2H, Ar-H), 7.07–7.12 (m, J = 8.78 Hz, 2H, Ar-H), 6.80–6.85 (m, 2H, Ar-H), 3.79 (s, 3H, -OCH_3), 3.17–3.23 (m, 2H, -CH_2), 3.09–3.15 (m, 2H, -CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 158.2, 154.5, 133.8, 130.5, 129.7, 127.3, 123.1, 121.5, 115.5, 113.7, 55.3, 35.8, 33.7.

*2-Benzyl-1H-benzo[d]imidazole (**11**)* [18]
Yield: 55%; White solid; mp: 260–264 ˚C; ^{1}H NMR (400MHz, DMSO-d_6) *δ*: 12.74 (s, 1H, NH), 8.08–8.16 (m, 2H, Ar-H), 7.58–7.66 (m, 2H, Ar-H), 7.46–7.54 (m, 1H, Ar-H), 7.17 (m, 2H, Ar-H), 7.09–7.13 (m, 2H, Ar-H), 3.34 (s, 2H, -CH_2). ^{13}C NMR (100MHz, DMSO-d_6) *δ*: 160.7, 151.5, 144.0, 135.1, 128.1, 122.8, 122.2, 121.5, 118.6, 114.4, 111.1, 55.3.

*2-(4-Methoxybenzyl)-1H-benzo[d]imidazole (**12**)* [18]
Yield: 85%; White solid; mp: 163–165 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.38 (br s, 1H, NH), 7.52 (br s, 2H, Ar-H), 7.17–7.24 (m, 4H, Ar-H), 6.87 (d, J = 8.54 Hz, 2H, Ar-H), 4.22 (s, 2H, -CH_2), 3.79 (s, 3H, -OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 157.9, 152.1, 134.5, 129.8, 128.6, 122.8, 115.2, 110.3, 55.9, 35.1.

*2-Phenyl-1H-benzo[d]imidazole (**13**)* [18]
Yield: 60%; Light yellow solid; mp: 288–290 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.92 (br s, 1H, NH), 8.15–8.22 (m, 2H, Ar-H), 7.64–7.71 (m, 1H, Ar-H), 7.46–7.59 (m, 4H, Ar-H), 7.16–7.27 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 150.5, 143.7, 134.5, 132.3, 129.8, 127.2, 126.6, 122.7, 121.4, 119.4, 110.2.

*2-(4-Methoxyphenyl)-1H-benzo[d]imidazole (**14**)* [18]
Yield: 48%; White solid; mp: 224–226 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.74 (s, 1H, NH), 8.08–8.16 (m, 2H, Ar-H), 7.58–7.66 (m, 1H, Ar-H), 7.46–7.54 (m, 1H, Ar-H), 7.17 (m, 2H, Ar-H), 7.09–7.13 (m, 2H, Ar-H), 3.34 (s, 3H, -OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 161.4, 152.3, 145.3, 135.3, 129.4, 127.5, 122.7, 121.6, 118.8, 113.2, 110.6, 55.2.

*4-methyl-2-phenethyl-1H-benzo[d]imidazole (**15**)* [21]
Yield: 53%; White solid; mp: 229–230 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.47 (dd, J = 3.15, 5.97 Hz, 2H, Ar-H), 7.15–7.11 (m, 6H, Ar-H), 3.18–3.12 (m, 4H, -CH_2), 2.12 (s, 3H, -CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 157.8, 145.6, 138.4, 134.2, 129.9, 128.7, 127.3, 122.5, 121.2, 119.6, 110.7, 34.8, 26,7, 21.3.

4-Methyl-2-phenyl-1H-benzo[d]imidazole (**16**) [20]
Yield: 86%; White solid; mp: 248–249 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.71 (s, 1H, NH), 8.24–8.18 (m, 2H, Ar-H), 7.57–7.46 (m, 3H, Ar-H), 7.34 (d, J = 6.8 Hz, 1H, Ar-H), 7.10 (s, 1H, Ar-H), 6.99 (d, J = 7.12 Hz, 1H, Ar-H), 2.58 (s, 3H, -CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 159.2, 148.4, 138.5, 133.1, 129.7, 128.9, 127.8, 126.3, 124.1, 121.7, 111.6, 21.4.

2.2. Biological Activity Studies

2.2.1. MTT Assay

Estrogen receptor-positive MCF-7 human breast cancer cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator. The cytotoxic activity of the eight synthesized compounds was evaluated using the standard MTT assay. Cells were seeded in 96-well plates at a density of 3 × 10^4 cells per well (n=3 replicates) and allowed to attach for 24 hours. Cell counts and viability were determined using a Cedex XS device (Roche, Mannheim, Germany), which measured cell suspension parameters via an automated Smart Slides system. Subsequently, cells were treated with increasing concentrations (1 μM, 10 μM, 50 μM) of the test compounds and incubated for 48 and 72 hours. Following the incubation period, MTT reagent was added to each well, and plates were further incubated for 4 hours. After removing the medium, 100 μL DMSO was added to solubilize the formazan crystals, and absorbance was measured at 570 nm using a microplate reader (μQuant, BioTek Instruments, Vermont, USA). The reduction of tetrazolium salt to formazan, a yellow-colored compound, indicated mitochondrial metabolic activity. The IC_{50} values (concentrations inhibiting 50% of cell viability) were determined using a logarithmic dose-response curve plotted in Microsoft Excel [22].

2.2.2. mTOR Activity

Chemical and Reagent

MCF-7 cells (HTB-22) were obtained from the American Type Culture Collection (ATCC, USA). The cell culture medium Dulbecco's Modified Eagle Medium (DMEM), along with penicillin-streptomycin, was purchased from Gibco (New York, NY, USA). Fetal bovine serum (FBS) and trypsin-EDTA were supplied by Biological Industries (Israel).

Experimental groups

The cell culture was performed in DMEM/F12 containing 10% FBS, 1% penicillin-streptomycin, and L-glutamine at 37 °C in 5% CO_2 incubator. When cell confluence reached 80%, the subculture was carried out. In 6-well plates, experimental cells were cultured at a density of 2 x 10^4 cells per well and grown to 80%-90% confluence [23]. Cells were divided into six groups: control; Rapa (20 μM); 7 (10,84 μM), 10 (6,40 μM); 11 (6,82 μM); and 12 (11,57 μM). All cells were incubated for 48 h at 37 °C in a 95% air, 5% CO_2 environment.

Experimental Design

Cells were cultured in DMEM/F12 medium containing 10% FBS, 1% penicillin-streptomycin, and L-glutamine at 37 °C in a 5% CO_2 incubator. When cell confluence reached 80%, subculturing was performed.

For experimental treatments, MCF-7 cells were seeded in 6-well plates at a density of 2×10^4 cells per well and grown to 80–90% confluence. Cells were then divided into six groups:

- Control (untreated)
- Rapamycin (Rapa, 20 μM, positive control)
- Compound 9, Compound 10, Compound 14, Compound 15

Cells were incubated for 48 hours at 37 °C under 95% air and 5% CO_2 conditions.

Measurement of mTOR activity

The effect of the synthesized compounds on mTOR signaling was assessed using a colorimetric cell-based enzyme-linked immunosorbent assay (ELISA). The phosphorylation level of mTOR at Ser2448, a key marker of mTORC1 activation, was measured according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Newman-Keuls multiple comparison test or unpaired t-test where appropriate. Statistical significance was defined as $P < 0.05$. Analyses were performed using GraphPad Prism (Prism 5.0; GraphPad Software, San Diego, California, USA).

3. Results and Disscussion

3.1. Chemistry

The targeted 2-substituted benzimidazoles (fig. 3) were synthesized using a highly efficient one-pot cyclodehydration method, in which readily available carboxylic acids (3–8) were reacted with 1,2-phenylenediamine derivatives (1 and 2) in the presence of polyphosphoric acid (PPA) acting as both catalyst and solvent. This approach, adapted from a previously reported method, is illustrated in Scheme 1.

For the synthesis of compounds 9–16, cyclodehydration reactions were performed on benzimidazole derivatives featuring various substituents, including phenyl, benzyl, and phenethyl groups, with the core structure being either unsubstituted or 4-methyl-substituted. These reactions yielded the corresponding final products (9–16) with efficiencies ranging from 48% to 86%. The chemical structures of the synthesized compounds were confirmed by ^{1}H NMR and ^{13}C NMR spectral analyses, which were consistent with the proposed structures.

3.2. Biological Activity Studies

Due to concerns such as drug toxicity and adverse effects, a more detailed investigation of various signaling pathways is essential for identifying novel targets in cancer therapy. The PI3K/Akt/mTOR signaling pathway plays a crucial role in multiple diseases, including breast and ovarian cancer. While this pathway regulates growth, proliferation, and cell survival under normal physiological conditions, its aberrant activation is a hallmark of cancer. Moreover, the PI3K/Akt/mTOR cascade interacts with key intracellular pathways, such as AMP-activated protein kinase (AMPK), Wnt/β-catenin, and mitogen-activated protein kinase (MAPK). Investigating the effects of newly developed anti-cancer agents on these pathways can provide

valuable insights into their therapeutic potential. In this context, we conducted in vitro studies to assess the mTOR inhibitory activity of the synthesized compounds.

3.2.1. MTT

The cytotoxic effects of the synthesized compounds on the MCF-7 cell line were evaluated at 48 and 72 hours using the MTT assay (Table 1). The IC_{50} values of the tested compounds were determined, and compounds 10 and 15, which exhibited the lowest IC_{50} values at both time points, were selected for further studies. A visual inspection suggested that the presence of an ethylene linker between the benzimidazole and phenyl moieties significantly influences the inhibitory potential of compounds 9, 10, and 15 compared to other derivatives. Notably, the introduction of a methyl substituent at the 4-position of the benzimidazole ring enhanced the antiproliferative activity of compound 15, which contains the ethylene linker.

Table 1. IC_{50} Values of Synthesized Compounds Measured 48 and 72 Hours After MTT Assay

Compounds	IC_{50} (µM)	
	48 h	72h
9	11.86	10.76
10	6.63	5.52
11	26.58	25.78
12	20.21	20.10
13	38.75	37.68
14	16.40	15.63
15	5.28	4.62
16	19.21	18.55

3.2.2. Evaluation of mTOR kinase inhibitory activities of the compounds *in vitro* MCF-7 cell lines

To investigate the inhibitory effects of compounds 10 and 15 on mTOR kinase, we quantified the phosphorylation levels of mTOR at Ser2448 in MCF-7 cells following 48-hour treatment. As shown in Figure 4, both compounds significantly reduced mTOR phosphorylation at Ser2448 compared to the control. MCF-7 cells were incubated with 0.1% DMSO (control), compounds 10 and 15, and rapamycin for 48 hours. The phosphorylation status of mTOR at Ser2448 was quantified based on OD values at 450 nm and expressed as mean ± SEM (n=6).

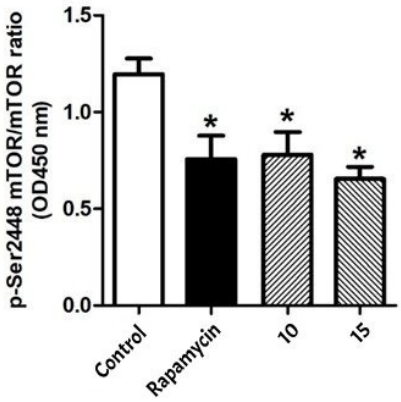


Figure 4. Effects of the compounds on the phosphorylation of mTOR at Ser2448 in MCF-7 cells. RAPA, rapamycin. *Statistically significant difference from the control group (Student's t-test; $P < 0.05$).

The results demonstrated that compounds 10 and 15 effectively reduced the p-mTOR (Ser2448)/mTOR ratio in MCF-7 cells. Similarly, the positive control, rapamycin, also suppressed mTOR activity, confirming the assay's reliability. These findings provide the first evidence that the newly synthesized benzimidazole-based compounds 10 and 15 exhibit inhibitory effects on aberrantly activated mTOR signaling in MCF-7 breast cancer cells.

4. Conclusion

In this study, a series of novel benzimidazole derivatives were designed and synthesized as potential mTOR inhibitors for breast cancer treatment. The *in vitro* cytotoxicity of these compounds was evaluated using the MTT assay against the MCF-7 cell line, where compounds 9, 10, 14, and 15 exhibited the most potent IC_{50} values. These findings provide a strong foundation for further optimization of these compounds as potential anticancer agents.
Subsequent biological activity studies focused on compounds 10 and 15, revealing significant mTOR inhibitory effects, particularly for compounds 10 and 15. These results highlight benzimidazole as a promising therapeutic scaffold for the development of targeted anticancer agents. Given their mTOR-inhibitory properties, benzimidazole-based derivatives could serve as valuable candidates for long-term anticancer drug development.
Among the tested compounds, compound 15 demonstrated particularly strong potential for further development as a novel mTOR inhibitor in breast cancer treatment. These findings contribute to a deeper understanding of mTOR-targeted therapy and reinforce the importance of continuous research and clinical evaluations to advance benzimidazole derivatives as future therapeutic agents.

Ethics in Publishing

There are no ethical issues regarding the publication of this study

Author Contributions

CRediT authorship contribution statement, Omer Faruk Col and Ronak Haj Ersan: Conceptualization, Data curation, Writing – original draft, Methodology, Validation, Formal analysis, Visualization, Investigation., Writing – review & editing

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