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RESEARCH ARTICLE

Using the MTT Cell Viability Test and Immunocytochemistry Techniques, Benzimidazole's Cytotoxicity and Apoptosis Activation in Mouse 4T1 Cell Culture

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Abstract

Cancer is known as a major health problem globally. Breast cancer is the most common malignant tumor and metastasis continues to be the main cause of poor prognosis. Despite recent advances in cancer treatment, the success of metastatic breast cancer treatment is not at the desired level. Among the anticancer drugs discovered in recent years, various benzimidazole derivatives have attracted attention in the development of anticancer agents due to their various biological activities and clinical applications. The aim of this study was to evaluate the antiproliferative activity of synthesized benzimidazole derivative SA-61 in 4T1 breast cancer cell line in comparison with abemaciclib, which is approved by FDA for the treatment of breast cancer. The antiproliferative activity and apoptotic effect of SA-61 were examined using MTT and immunohistochemical methods, respectively. According to MTT results, compound SA-61 showed antiproliferative activity in 4T1 cells in a dose-dependent manner, but this effect was lower than abemaciclib. Although further studies are needed to specifically identify the compounds involved in its anti-cancer activity, our findings suggest that SA-61 inhibits the proliferation of 4T1 cells and its effect is dose dependent.

Key Words: Benzimidazole, Casp-3, cytotoxic activity, 4T1 cell line

Benzimidazolün Fare 4T1 Hücre Kültüründeki Apoptoz Aktivasyonunun ve Sitotoksisitesinin MTT Hücre Canlılık Testi ve İmmunsitokimya Yöntemleriyle Araştırılması

Öz

Kanser, küresel olarak önemli bir sağlık sorunu olarak bilinmektedir. Meme kanseri en yaygın kötü huylu tümördür ve metastaz kötü prognozun başlıca nedeni olmaya devam etmektedir. Son yıllarda kanser tedavisindeki gelişmelere rağmen, metastatik meme kanseri tedavisinin başarısı istenen düzeyde değildir. Son yıllarda keşfedilen antikanser ilaçları arasında, çeşitli biyolojik aktiviteleri ve klinik uygulamaları nedeniyle çeşitli benzimidazol türevleri antikanser ajanı geliştirmede dikkat çekmiştir. Bu çalışmanın amacı, 4T1 meme kanseri hücre hattında, sentezlenen benzimidazol türevi olan SA-61'in antiproliferatif aktivitesini, FDA tarafından meme kanseri tedavisi için onaylanan abemaciclib ile karşılaştırmalı olarak değerlendirmektir. SA-61'in antiproliferatif aktivitesi ve apoptotik etkisi sırasıyla MTT ve imunohistokimyasal yöntemler kullanılarak incelendi. MTT sonuçlarına göre SA-61 bileşiği 4T1 hücrelerinde doza bağlı bir şekilde antiproliferatif aktivite gösterdi fakat bu etki abemaciclibe kıyasla daha düşüktür. Kanser karşıtı aktivitesinde rol oynayan bileşiklerin spesifik olarak belirlenmesi için daha fazla çalışmaya ihtiyaç duyulmasına rağmen, bulgularımız SA-61'in 4T1 hücrelerinin çoğalmasını engellediğini ve etkisinin doza bağlı olduğunu göstermektedir.

Anahtar Kelimeler: Benzimidazol, Casp-3, sitotoksik aktivite, 4T1 hücre hattı

INTRODUCTION

In terms of both prevalence and cancer-related death among women, breast cancer is the most common cancer diagnosed globally (1). There is currently no accurate way to forecast the development of metastatic lesions, and current treatments for metastatic breast cancer are unsuccessful. Finding novel biomarkers and treatment targets for breast cancer requires a deeper comprehension of the processes behind breast cancer metastasis (2). A mouse initial breast tumor that developed in a BALB/c mouse raised and breastfed by a C3H female mouse is a subclone of 4T1 breast cancer, a triple-negative subtype of breast cancer (3). 4T1 cells are diverse clonal subpopulations with unique shape, behavior, and gene expression profiles, in contrast to previous cancer models (4). Even though some patients may not respond completely to chemotherapy, it is still the cornerstone of treatment for triple-negative breast cancer because the therapeutic targets are unknown (5). Thus, it is imperative to find a possible target for triple-negative breast cancer treatment.

Because of their varied biological activity and therapeutic uses, several benzimidazole derivatives have garnered interest in the creation of anticancer agents among the anticancer medications found in recent years (6). Benzimidazole is a great scaffold for the creation of anticancer drugs because of its distinct core structure and low toxicity. Because of their structural resemblance to nucleosides, benzimidazole and its derivatives exhibit potent anticancer action (7). Furthermore, benzimidazoles can bind to many pharmacological targets implicated in the advancement of cancer and function as hydrogen donors or acceptors. In the literature, there are numerous anticancer medications with benzimidazole cores that have garnered a lot of interest (Figure 1).

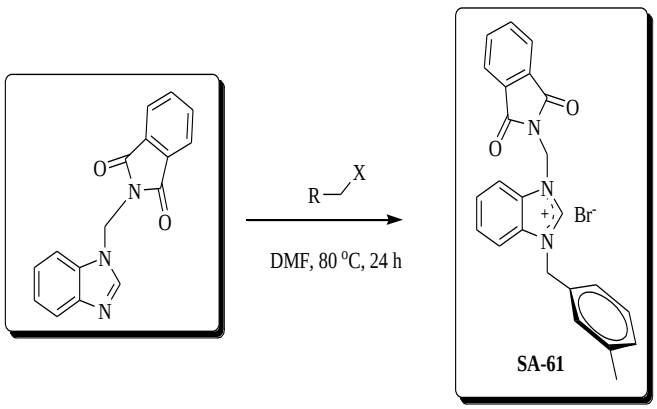

Figure 1. Synthesis of a compound including benzimidazole core, SA-61.

Abemaciclib works well against a variety of solid tumors, such as melanoma, liposarcoma, esophageal and non-small cell lung cancers, and breast carcinoma. Abemaciclib is presently undergoing clinical trials for additional solid tumors and has FDA approval for the treatment of breast cancer (8). Abemaciclib's capacity to suppress growth *in vitro* and *in vivo* provides justification for its use (9). In light of this, the current work sought to compare the cytotoxic effect and apoptosis activation of SA-61, a benzimidazole derivative, to Abemaciclib in the 4T1 mouse breast cancer cell line.

MATERIAL AND METHODS

General Synthesis of 1-(N-Phthalimidomethyl)-3-(3-methylbenzyl) benzimidazolium Bromide, SA-61

A solution of benzimidazole (1 mmol) and potassium hydroxide (1 mmol) was prepared in 60 mL of ethanol. The reaction mixture was stirred at room temperature for 1 hour, after which 3-methylbenzyl chloride (1 mmol) was gradually added. The solution was then refluxed for 6 hours and allowed to cool to room temperature. The resulting potassium chloride precipitate was removed by filtration, and the solvent was evaporated. The intermediate product, 1-(3-methylbenzyl) benzimidazole, was recrystallized, washed multiple times with diethyl ether, and dried under vacuum. Next, 1-(3-methylbenzyl) benzimidazole (1.29 g, 1 mmol) was dissolved in 4 mL of dried DMF, and N-(bromomethyl) phthalimide (1.39 g, 1 mmol) was added dropwise. The reaction mixture was stirred at 80 °C for 24 hours under an argon atmosphere. Upon completion, DMF was removed under reduced pressure, and 15 mL of diethyl ether was introduced. The solid was collected, washed twice with diethyl ether (2 × 15 mL), and dried under vacuum. Finally, the purified product was crystallized in an ethanol/diethyl ether (3:1) mixture at room temperature, yielding a white solid (10).

4T1 Cells

4T1 breast cancer in mice as previously documented, 4T1 cells were cultivated in RPMI 1640 supplemented with 10% FBS, 100 µM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate (complete medium). The cells were procured from ATCC, Manassas, VA, USA. There have been prior reports of the production of 4T1 cells (A8) lacking GM-CSF. In T-75 tissue culture flasks, 4T1 cells were cultivated in full media to create 4T1 cell culture supernatant (4T1-sup). Cell-free supernatants were obtained by centrifugation at 1200 rpm for 10 minutes after the medium's color began to become somewhat yellow. Amicon Ultra-15 centrifugal filters were used to concentrate cell-free supernatants.

The Cytotoxic Activity Studies

The process outlined in the literature was followed to conduct cytotoxic activity investigations of compound SA-61 and abemaciclib as a positive control medication (11,12). The 4T1 mouse breast cancer cells (ATCC CRL-2539) were cultivated in RPMI with 1% glutamax and 10% fetal bovine serum (FBS) added. Cells were seeded into sterile 96-well plates at a density of 5×10^3 cells/well. For 48 hours, the cells were exposed to the chemicals at doses of 200, 100, 50, 25, and 12.5 µM. After adding the MTT stock solution (50 µL, 5 mg/mL) to the plate wells, the plates were incubated for two more hours. The Promega Elisa plate reader instrument was used to detect absorbance levels at 590 nm. GraphPad Prism Software 5 was used to calculate the IC_{50} values.

Immunocytochemistry Staining

Prior to cell staining, the cells were incubated in PBS at 37 °C for 15 minutes after the top medium in the culture medium was removed. Following the removal and disposal of the

PBS, the cells were fixed for 30 minutes at 4 °C in 70% ethanol. The coverslips were incubated in a 0.5% H_2O_2 solution made in methanol for 10 minutes after being left in PBS for 10 minutes. They were then rinsed three times for 5 minutes each with distilled water. Following a 30-minute incubation period in 4N HCl, the samples underwent three 5-minute PBS washes. After that, a 20-minute protein block was applied. HRP-conjugated goat anti-rabbit antibody (ImmPRESS® MP-7451) was utilized as the secondary antibody for caspase-3 detection, whereas Cleaved Caspase-3 (Asp175) (Cell Signaling, 9661) was employed as the primary antibody at a 1:400 dilution. Following washing, coverslips were counterstained with Mayer hematoxylin and treated for equal amounts of time with DAB chromogen. They were successively exposed to increasing concentrations of alcohol (50, 80, and 100 percent) before being allowed to dry. Lastly, entellan mounting on slides prepared the preparations for analysis. Cells were examined for immunopositivity at 40× magnification (bar 20 µm) in order to assess the staining.

RESULTS

Cytotoxic activity studies of benzimidazole SA-61 were evaluated against the mouse breast cancer cell line 4T1. For comparative purposes, the cytotoxicity of abemaciclib (a targeted anticancer drug used in the treatment of advanced or metastatic breast cancer) was evaluated under the same conditions. The MTT assay was used in cell culture studies to determine the relationship between cell viability under various treatments. Compounds were evaluated for their potential anticancer activity using the IC50 value, which is the dose of the molecule that caused a 50% decrease in survival as determined by the MTT assay. The results of the viability rates of 4T1 cells cultured with abemaciclib and SA-61 are given in Chart 1. Accordingly, when different concentrations of abemaciclib (12.5, 25, 50, 100, 200 µM) and SA-61 (12.5, 25, 50, 100, 200 µM) were applied to 4T1 cells for 48 hours, it was determined that the IC50 dose reached corresponded to the dose required to kill half of the cells (Table 1). SA-61 showed an IC50 value of 98.25 µM against 4T1 cell line. This result indicates that SA-61 shows moderate anticancer activity in 4T1 cells. Abemaciclib showed an IC50 value of 14.84 µM against 4T1 cell line. This result revealed that abemaciclib showed a higher anticancer effect compared to SA-61 in 4T1 cells. Abemaciclib exhibited a more potent anticancer profile compared to SA-61 by showing a lower IC50 value in the 4T1 cell line. This lower value indicates that SA-61 is less effective in 4T1 cells and has less potential to inhibit breast cancer cells. Cellular proliferation was measured for mouse breast cancer 4T1 cells. Mouse breast cancer cells exposed to various concentrations of SA-61 and abemaciclib were observed at the 48-h time point. Significant differences were detected in 4T1 cells treated with different concentrations of SA-61 and abemaciclib. It was found that SA-61 had the highest antiproliferative effect at a concentration of 200 µM. Significant differences were also observed for abemaciclib in terms of cytotoxic effects caused by the doses. It is seen in Figure 2 that abemaciclib significantly inhibited the growth of 4T1 cells at concentrations of 200, 100 and 50 µM.

Chart 1. Cell viability ratio depending on concentrations of compound SA-61 and abemaciclib

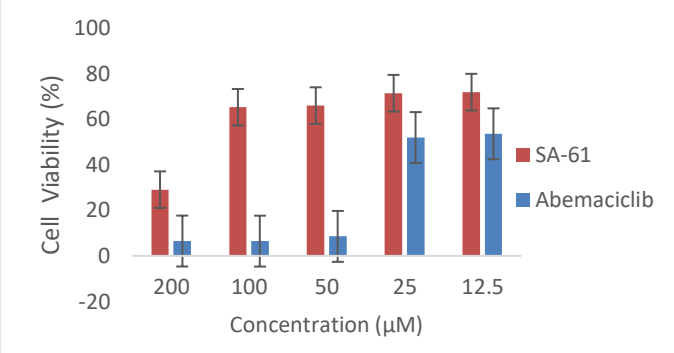

Table 1. IC_{50} results for compounds against mice cancer cell line

Compounds	IC_{50} (µM)
	4T1
SA-61	98.25
Abemaciclib	14.84

4T1 mouse mammary tumor cells were treated with SA-61 for 48 hours in order to investigate the apoptotic effects of the substance. Immunocytochemical staining was utilized to evaluate alterations in caspase-3 enzyme activity. 4T1 mouse mammary tumor cells showed increased caspase-3 activity in response to 48 hours of incubation with 98.25 µM SA-61, a 50% growth suppression dosage, as compared to untreated cells (Figure 2). These results imply a possible link between SA-61-induced apoptosis and caspase-3 activation.

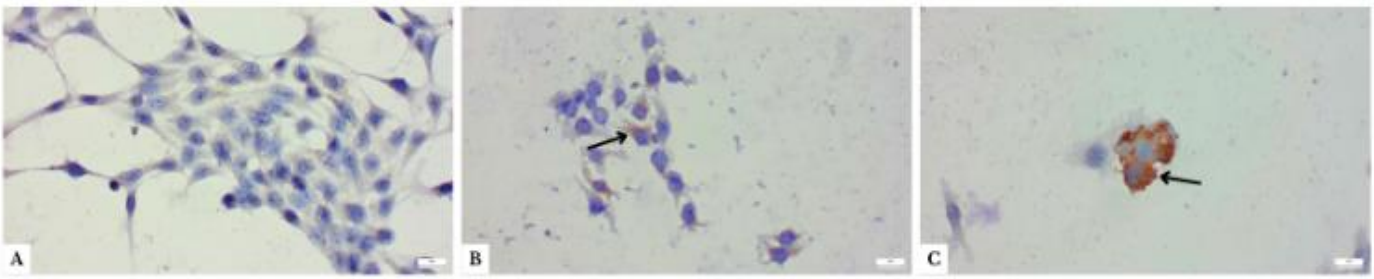

Figure 2. Immunoreaction of 4T1 mouse mammary tumor cells with cleaved-caspase3 antibody. A. Control (untreated) B. Abemaciclib (IC_{50}) C. SA-61 (IC_{50}). Nuclei are stained blue, positive cells are stained brown (arrow). Bar: 20 µm.

DISCUSSION AND CONCLUSION

With a high number of new cases and the second-highest number of fatalities, breast cancer continues to be a major cause of morbidity and mortality among women despite advancements in detection and treatment (13,14). Chemotherapy, surgical resection, and radiation therapy are common treatment approaches for primary breast cancer; however, metastatic breast cancer presents a considerable challenge and is a major cause of cancer-related mortality (15). Surgery, chemotherapy, radiation, endocrine therapy, targeted therapy, and other similar methods are considered conventional therapies for breast cancer (16).

Abemaciclib is a suitable option because it can cause atypical cell death and may also encourage autophagy and apoptosis. Abemaciclib employs H+ transport to cause lysosomal acidification. Through a distinct molecular mechanism, the impact on V-ATPase seems to result in lysosomal malfunction and ultimately cell death, as well as lysosomal growth brought on by H_2O influx (17). Abemaciclib has been shown to induce this type of cell death phenotype in MDA-MB-231 triple-negative breast cancer cells, A549 non-small lung carcinoma cells, and prostate cancer cells (18).

Although caspases are involved in apoptosis, prior research has discovered that caspase-3 is also involved in the autophagic process (19). In human apoptotic endothelial cells under nutritional deprivation, caspase-3 reoriented autophagic vacuoles toward the cell membrane, promoting their extracellular transit (20). Apoptotic volume decrease, a geometric predictor of cell disintegration into apoptotic bodies, may be influenced by the transit of these massive autophagic vacuoles (18). These results imply that caspase-3 is a node that controls how the apoptotic and autophagic pathways interact (20).

Benzimidazole compound had potent apoptotic effects in 4T1 cells, according to our investigation. Although the control group did not exhibit caspase-3 staining, cells treated with abemaciclib, which served as a positive control, only mildly and sparingly showed favorable results. On the other hand, cells treated with benzimidazole compound showed strong caspase-3 positivity, suggesting a substantial apoptotic response. This result is in line with earlier research that demonstrated that DMDD increases caspase-3 and -9 activation to start the apoptotic process (21). Similarly, it is recognized that natural substances like resveratrol and piperine have anticancer effects via apoptotic processes. Piperine inhibits cell proliferation by inducing cell cycle arrest in the G2/M phase and initiates apoptosis through caspase-3 activation. However, by stopping cells in the S phase, resveratrol exhibits lethal effects on cancer cells (22, 23). Furthermore, it has been demonstrated that CQ triggers cell death by activating caspase-9 and caspase-3 and activates the mitochondrial apoptotic pathway, which results in the release of cytochrome c (24). According to these findings, benzimidazole derivatives can also encourage the death of tumor cells by apoptosis and could be used as a possible anticancer medication.

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CONFLICT OF INTEREST

"There is no conflict of interest to be declared by the authors."

AUTHOR CONTRIBUTIONS

"All analyses, writing and final checks of the study were performed equally among the authors."

ETHICAL STATEMENT

"There is no need for an ethics committee in the study."

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