Clinical and Experimental Health Sciences

Amcasertib Increases Apoptosis While Decreasing Invasive and Migrating Abilities in Breast Cancer Stem Cells

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ABSTRACT

Objective: A relationship exists between breast cancer stem cells (BCSCs) and the chemo-resistance and recurrence of aggressive breast tumors. Amcasertib is a small chemical compound and multiple kinase inhibitor that inhibits downstream Nanog and other cancer stem signaling pathways in cancer stem cells by targeting several serine-threonine kinases. In this study, we aimed to investigate the cytotoxicity and anticancer effects of Amcasertib on BCSCs, gaining insight into the targetability of BCSCs.

Method: We used the combined xCELLigence-Real-Time Cell Analyzer (RTCA) equipment to analyze cytotoxicity and cell proliferation. We detected the IC_{50} dosages of Amcasertib at 24, 48, and 72 hours and examined its effects on apoptosis, cell cycle, invasion, and migration over 48 hours. We used flow cytometry for assays of apoptosis and cell cycle, and the CytoSelect 96-well Cell Migration and Invasion Assay Kit for evaluating invasion and migration.

Results: Our results showed that Amcasertib has cytotoxic properties, with an IC_{50} dosage of 1.9 µM at the 48th hour. In addition, Amcasertib significantly induced apoptosis in BCSCs, despite not affecting the cell cycle. Moreover, Amcasertib decreased BCSCs' invasion and migratory properties, part of epithelial-mesenchymal transition (EMT).

Conclusion: In conclusion, our findings provide crucial information for understanding the potential of Amcasertib in targeting BCSCs. In addition, we suggest that Amcasertib could be a beneficial drug for breast cancer treatment by targeting BCSCs.
Keywords: Amcasertib, small molecule inhibitor, stemness kinases, breast cancer stem cell

1. INTRODUCTION

Breast cancer is a significant global health issue because of its high rates of illness and death among women. In 2020, there were approximately 2.3 million new cases and 684,996 reported deaths, highlighting the high prevalence of the disease among women (1,2). The spread of cancer to other organs, known as metastasis, is responsible for more than 90% of deaths from breast cancer (3,4). Breast cancer, like other malignancies in humans, is both a hereditary and a sporadic disease (5). The most common causes of hereditary breast cancer are mutations in the BRCA1 and BRCA2 genes (6). High-risk genes for breast cancer include TP53, PTEN, STK11/LKB1, and CDH1, although these genes are associated with an increased rather than decreased chance of developing cancer in carriers (7). Histology and molecular features are used to classify the many different types of tumors that comprise breast cancer (8). Genetic profiling and immunohistochemical research have led to the classification of breast cancer into five subtypes: "luminal A," "luminal B," "HER2 overexpression," "basal-like," and "normal breast-like"(9). The gene expression patterns, clinical features, therapeutic response, and prognosis of these breast cancer molecular subgroups are distinct (10).

Cancer stem cells, which are also called tumor-initiating cells, can be found in a small number of malignancies. These cells can proliferate and differentiate (11,12). Biological activities of cancer stem cells, such as recurrence, metastasis, heterogeneity, multidrug resistance, and radiation resistance, are regulated by pluripotent transcription factors such as OCT4, Sox2, Nanog, KLF4, and c-Myc, which lead to treatment failure (12).

"Targeted therapies" are cancer treatments that are meant to kill only cancer cells without hurting any healthy tissue. Yet, conventional chemotherapy has the potential to kill healthy cells along with cancer cells (13). During the last 30 years, the FDA has approved 18 small-molecule drugs for various types of breast cancer, including those that are ER-positive, HR-positive, HER2-positive, BRCA-mutated, and PIK3CA-altered. These drugs; function as a microtubule-stabilizing agent, a prodrug of 5-FU, a DNA topoisomerase II inhibitor, an aromatase inhibitor, an estrogen receptor (ER) inhibitor,

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an estrogen receptor (ER) antagonist, a cyclin D-cyclin-dependent kinase 4/6 inhibitor, a HER2 and EGFR inhibitor, a HER2 inhibitor, a PARP inhibitor (14); on the other hand, there is no evidence to suggest that they are deliberately aiming for BCSCs. However, several small-molecule inhibitors and immunotherapeutic agents targeting BCSC have been reported in preclinical and clinical trials (15). These molecules target stemness markers (CD44, ALDH1, CD133, etc.), HIF family members inhibitors, EGFR/HER2 signaling axis, and several signaling pathways contributing stemness such as Notch, PI3K/AKT/mTOR, Wnt/β-Catenin, and Hedgehog (16).

Nanog is a stemness-related transcription factor that helps to maintain embryonic stem cell pluripotency and self-renewal. It is used as a marker to detect cancer stem cells and may also be used to identify these cells (17). Nanog is overexpressed in a variety of cancers, including those of the brain, breast, colon, ovary, and prostate (18). Nanog overexpression is associated with a more advanced stage of cancer, a lack of differentiation, a shorter overall survival, and the emergence of resistance to treatment. As a result, Nanog has been identified as a potential therapeutic target for treating cancer (19). In 2017, research into amcasertib, a first-in-class CSC-specific small-molecule multi-kinase inhibitor created by Boston Biomedical Inc., began in advanced adenoid cystic carcinoma (ACC) and advanced head and neck tumors. The orally active agent, well tolerated, works by inhibiting downstream cancer stem signaling pathways, including Nanog, by targeting several serine-threonine kinases (20,21). Amcasertib (Figure 1) is a potential anticancer drug investigated with just clinical trials, except for two preclinical studies (22,23).

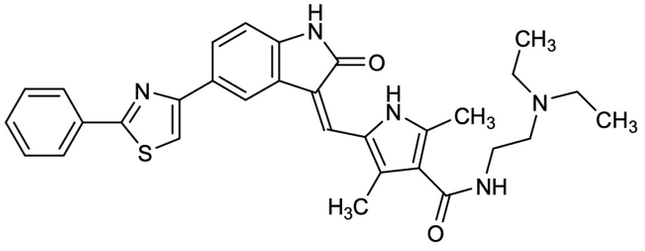

Figure 1. *The structure of amcasertib. Molecular formula is (C31H33N5O2S). Chemical name is N-[2-(diethylamino)ethyl]-2,4-dimethyl-5 {[2-oxo – 5-(2-phenyl-1,3-thiazol-4-yl)-1,2-dihydro-3H-indol – 3-ylidene]methyl}-1H-pyrrole-3 carboxamide (49).*

In this study, we aimed to investigate the cytotoxic, apoptotic, anti-invasion, and anti-migration effect of the multi – stemness kinase inhibitor Amcasertib directly on breast cancer stem cells (BCSC) in vitro.

2. METHODS

2.1. Cell Line and Chemicals

The breast cancer stem cell line (BCSC, #36102-29) was purchased from Celprogen (Torrance, United States). As stated in the datasheet, clonal selected stem cell line was derived from source of human breast cancer tissue (Triple negative; ER – negative, PR – negative, and HER2 – negative) and its positive markers are CD133, CD44, SSEA3/4, Oct4, Tumorigenicity (<1000 cells), Alkaline Phosphatase (ALP), Aldehyde Dehydrogenase (ALDH), Telomerase, Sox2 (24). BCSC cells were cultured in Human Breast Cancer Cell Line Complete Media with Serum (#M77002-07S) under a humidified atmosphere of 5% CO_2 in air at 37°C. Amcasertib (Cat no: S8572) purchased from Selleckchem (Planegg, Germany) was dissolved in DMSO and stored at – 80°C based on the manufacturer's recommendation.

2.2. Proliferation and Cytotoxicity Tests

Analyses of cytotoxicity and cell proliferation were carried out using the combined xCELLigence-Real-Time Cell Analyzer (RTCA) equipment, which allows for the continuous monitoring of cellular activities. To examine cell growth, a 96-well e-plate was seeded with densities ranging from 1×10^6 to 2×10^3 cells/ml. The e-plate was mounted on the RTCA system, and the culture of cells was allowed to continue for a full 96 hours. After incubation, the optimal cell proliferation numbers were found by choosing the required periods based on the cell index graphs found in the system. This was done after the cells had been allowed to proliferate. During the cytotoxicity test, the necessary cell number was seeded into 96-well plates after being determined by the cell proliferation test performed on the BCSC model. The microplates were then incubated in the RTCA system for 24 hours. After incubation, the cells were subjected to a treatment consisting of three repeats of amcasertib at a dosage ranging from 10 – 0.5 µM over 72 hours. The control group consisted of cells that had not been treated in any manner. After that, the cell index graphs that were produced in the system were used to estimate the IC_{50} dosages of amcasertib for the 24th, 48th, and 72nd hour time periods (25).

2.3. Apoptosis Test by Flow Cytometry

The Annexin V-FITC Apoptosis Detection Kit was used to analyze the apoptotic cell death of BCSC cells to amcasertib (BD Pharmigen, USA). BCSC cells in the number of cells identified as a result of the proliferation test were seeded in 6-well plates, and the microplates were then incubated for 24 hours. After incubation, the cells were subjected to a treatment with amcasertib at the IC_{50} dose and for the time specified. Untreated cells were used as control. Trypsin was used to detach the BCSCs, and then they were washed twice with PBS. After the stage of washing, one hundred microliters of binding buffer diluted one hundred times was applied to the cells. After the addition of 5 µl of FITC-Annexin V and 5 µl of propidium iodide (PI) antibody to the cells, while they were in the binding solution, the cells were left to incubate at room temperature and in the dark for 15 minutes. Following incubation, 400 µl of binding buffer was added to the cells, and then the cells were examined using CFlow Plus software on a flow cytometer (DB Accuri C6, Becton Dickinson, USA).

2.4. Cell Cycle Test by Flow Cytometry

Amcasertib's influence on the cell cycle was studied using a model of breast cancer stem cells and the BD Cycletest Plus DNA Kit, which was used following the protocol provided

for the kit. After seeding BCSC cells into 6-well plates and determining the cell number based on the results of a proliferation study, the plates were left to incubate for 24 hours. After incubation, BCSC cells were exposed to treatment with amcasertib at the dosage and period determined by the IC_{50}. Untreated cells were used as control. Trypsin was used to detach the BCSCs, and then they were washed twice with 1× PBS. The approach consisted of suspending the cells in 1 ml of buffer solution, centrifuging them at 300×g for 5 minutes, and then repeating this process twice. After adding 250 µl of Solution A to the cells and incubating them for 10 minutes at room temperature, the cells were taken out of the dish. After the incubation, 200 µl of Solution B was added to the cells. Subsequently, the cells were incubated again for 10 minutes at room temperature. After that, 200 µl of cold Solution C was added to the cells, and the cells were left to incubate on ice in the dark for ten minutes. Reading cells in flow cytometry allowed for the calculation of the percentages of cells in each stage of the cell cycle (BD Accuri C6, Becton Dickinson, USA).

2.5. Invasion and Migration Analysis

The CytoSelect 96-well Cell Migration and Invasion Assay Kit (Cell Biolabs, USA) was used to evaluate the effect of amcasertib on invasion and migration in a breast cancer stem cell model. The evaluation was performed according to the protocol provided with the kit. The IC_{50} dosage of amcasertib was administered to BCSC cells for 24 hours. We used the cells in the sample that had not been treated with amcasertib as a control group. After the treatment of the kit methodology to the control cells as well as the cells that had been treated with amcasertib, the relative fluorescence units (RFU) were measured using a fluorescent plate reader with a 485/538 nm filter.

2.6. Statistical Analysis

The results were analyzed with GraphPad Prism v.10.1.0 software using a two-tailed unpaired Student's t-test for calculating the *p*-value as the level of significance of comparison of treated and untreated cells. The p value < .05 was considered statistically significant. Data were presented as mean ± SD of three biological replicates.

3. RESULTS

3.1. Amcasertib Suppresses Breast Cancer Stem Cell Growth

As a result of the experiment involving cell proliferation, the optimal cell concentration in the logarithmic phase for BCSC cells was found to be 2×10^4 cells/ml. Amcasertib was shown to have a cytotoxic effect on BCSC cells, and the extent of that effect was found to be dependent both on the dosage administered and the amount of time that the treatment was allowed to take effect. In the BCSC cell line, the IC_{50} value of amcasertib was found to be 2.9 µM at 24 hours, 1.9 µM at 48 hours, and 1.8 µM at 72 hours. These values were established after the drug was tested for the appropriate period (Figure 2).

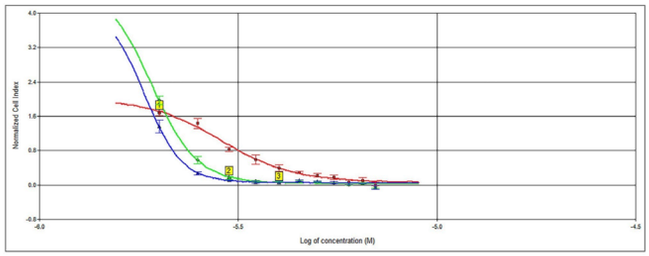

Figure 2. *The cytotoxic effects of Amcasertib in the BCSC cells. It shows dose-response curves for the 24th hour with "1" (red line), for the 48th with "2" (green line), and for the 72nd hour with "3" (blue line).*

3.2. Apoptosis is Induced in Breast Cancer Stem Cells by Amcasertib

After 48 hours of treatment with amcasertib at a concentration of 1.9 µM, a significant increase in apoptotic effect was observed in breast cancer stem cells (BCSCs). The percentage of total apoptotic cells in BCSCs rose from 18.6% in the control group to 97.2% in the alisertib-treated group, indicating a 5.24-fold increase compared to the control condition (Figure 3).

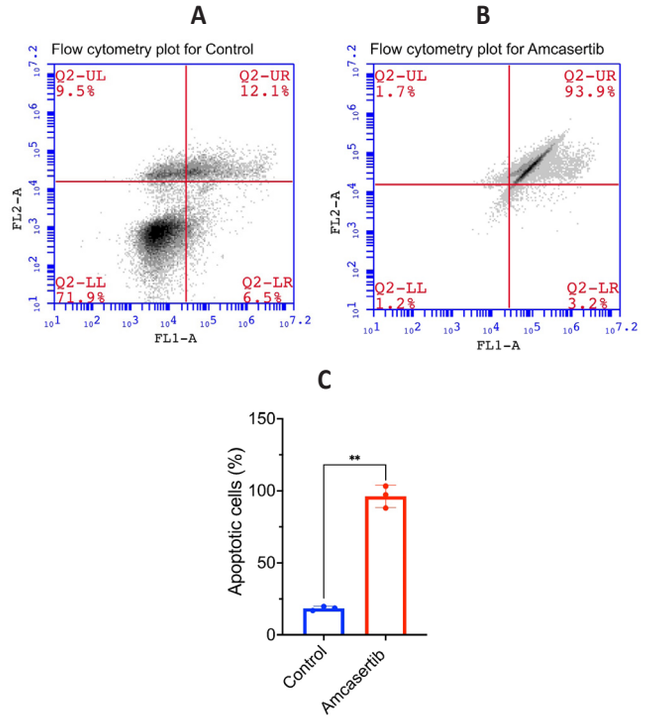

Figure 3. *Apoptotic effects of Amcasertib for 48 h in BCSC cells. Flow cytometry plots show apoptosis in cells without amcasertib treatment (A) and cells with amcasertib treatment (B) (FL2-A, PI fluorescence channel; FL1-A, FITC fluorescence channel; LL, live cells; LR, early apoptotoic cells; UR, late apoptotoic cells; UL, necrotic cells). C)The plot of the distribution percentage of apoptotic cells shows that apoptosis significantly increases in cells with amcasertib treatment compared to the cells without amcasertib treatment according to the two-tailed unpaired Student's t-test. Data shows as mean ± SD of n:3 replicates (**, p < .01).*

3.3. Cell Cycle Progression is Unaffected by Amcasertib in Breast Cancer Stem Cells

After 48 hours of treatment with amcasertib at the IC_{50} concentration of 1.9 µM, the proportion of breast cancer stem cells (BCSCs) in the G1 phase experienced a slight increase from 67.4% in the control group to 69.9% in the amcasertib-treated group. However, this increase was not statistically significant. (Figure 4).

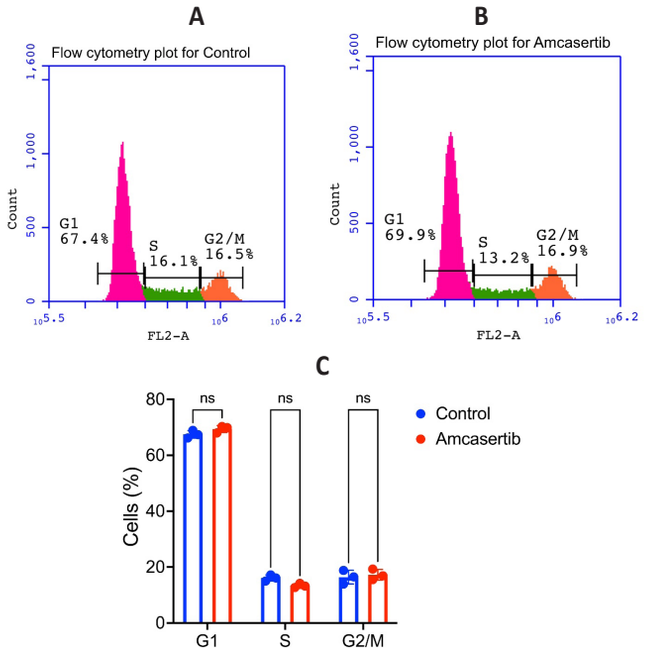

Figure 4. *Effects of Amcasertib on the cell cycle phases of BCSC cells for 48 hours. Flow cytometry plots show the distribution of cells without amcasertib treatment (A) and cells with amcasertib treatment (B) in cell cycle phases (FL2-A, PI fluorescence channel). C) The plot of the distribution percentages of cells in cell cycle phases shows no significance between cells with and without amcasertib treatment according to the two-tailed unpaired Student's t-test. Data shows as mean ± SD of n:3 replicates (ns, no significance).*

3.4. Amcasertib Reduces Invasion and Migration in Breast Cancer Stem Cells

Our investigation revealed a significant reduction in the cell invasion and migration activities of BCSCs subjected to a 48-hour treatment with 1.9 µM amcasertib (Figure 5).

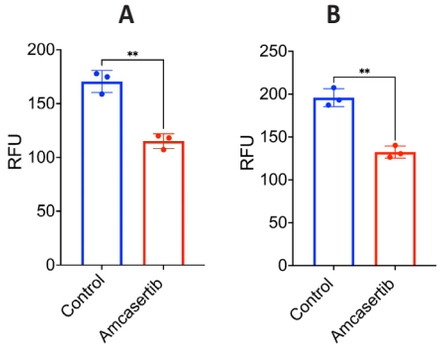

Figure 5. *Effects of Amcasertib on migration and invasion of BCSC cells for 48 hours. The plots of relative fluorescence units (RFU) for invasion (A) and migration (B) show that both invasion and migration significantly decrease in cells with amcasertib treatment compared to the cells without amcasertib treatment according to the two-tailed unpaired Student's t-test. Data shows as mean ± SD of n:3 replicates (**, p < .01).*

4. DISCUSSION

Since their identification in 2003, breast cancer stem cells (BCSCs) have been an important study issue for elucidating the malignancy of breast cancer, a complex disease with many etiology (26,27). Twenty percent of individuals with BCSCs show signs of chemoresistance and recurrence (28). One of the biggest challenges in treating breast cancer is the development of resistance to the drugs now in use; hence, efforts to develop a BCSC-targeted approach to combat this problem have been highlighted (29).

The anti-cancer effects of small molecules on breast cancer have been studied in vivo and in vitro, with many studies reporting that the small compounds specifically targeted BCSCs. Rather than using a dedicated BCSC cell line, researchers in these studies demonstrated the drugs' impact on BCSC by assessing alterations in the CD44+CD24- and/or $CD44^{+}CD24^{-}ALDH^{+}$ cell populations within murine or human breast cancer cell lines (triple negative or HR-positive), xenograft models, or patient tumor tissues (30–37). Nevertheless, in our research, we utilized directly a BCSC cell line, isolated from triple-negative breast cancer tissue, that has been verified by the manufacturer and was made available commercially. We explored the anticancer impact of amcasertib on BCSCs because, unlike other small medicines that target a particular cellular molecule, amcasertib targets selectively multiple cancer stemness kinases (20). Amcasertib has been studied extensively in individuals with advanced cancers other than breast cancer (phase I, phase Ib, phase Ib/II, and phase II trials all completed, NCT01781455, NCT02232633, NCT02279719, NCT02354898, NCT02483247, NCT02432326) (38–43).

Two distinct preclinical investigations have been conducted to assess the pharmacological impact of amcasertib. In the first study, focused on investigating the correlation between Iroquois-class homeodomain protein 4 (IRX4) and EGFR-tyrosine kinase inhibitor resistance, the inhibitory effects of amcasertib (10-0.3125 M, 1:2 serial dilution) for 48 hours on cell growth in gefitinib-resistant non-small cell lung cancer PC-9 cells (PC-9/GR) were observed in a dose-dependent manner (22). The second study, undertaken by our research team, aimed to explore the anticancer properties of amcasertib in ovarian cancer (23). Amcasertib's IC_{50} was found to be 2.9 µM after 24 hours, 1.9 µM after 48 hours, and 1.8 µM after 72 hours of treatment of BCSCs. Considering that other studies with data about triple negative BCSC population and using small molecule inhibitors may be seen much lower IC_{50} doses for relevant small molecule inhibitors. However, it may be based on being used triple-negative cell lines (TNBC) with a heterogeny population rather than a just BCSC niche. For example, exportin-1 or CRM1 inhibitor LFS-1107 was reported to exhibit an IC_{50} value of

40.80 nM in the MDA-MB-231 TNBC cell line for 72 hours (34). In a study investigating anticancer effects of Fluorine-Incorporated Gold I Compound (3F1) on human breast cancer cells by using commercial cell lines MDA-MB-231, MCF-7 (ER^+PR^+) and BCSC cell line (#36102-29, Celprogen), IC_{50} dose of 3F1 for 24 hours was reported 10.17 µM in BCSC, whereas 8.44 µM and 6.17 µM in MDA-MB-231 and MCF-7, respectively (44). Therefore, we'd like to point out that amcasertib exhibited an effective cytotoxicity on BCSCs. In addition, we found that amcasertib promoted apoptotic cell death with a significant increase over the control group. Thus, our current study exhibits in parallel our findings in the recent study demonstrating significant antiproliferative effects and apoptosis induction by amcasertib in ovarian cancer cells (MDAH-2774 and OVCAR-3) and ovarian cancer stem cells (OCSC). Nevertheless, we found that it did not lead to cell cycle arrest in BCSCs, like in OCSCs (23). Some studies emphasizing that different small molecule inhibitors targeted BCSC population in TNBC cells were reported to increase the sub-G1 cell population as depend on increasing apoptotic effect and induce G2/M phase arrest in MDA-MB-231 cells in a dose-dependent manner (35,36). We didn't evaluate sub-G1 cell population. However, abcasertib may have caused increasing in the sub-G1 cell population as depended on the strong apoptotic effect detected, although it exhibited an insignificant effect on the cell cycle phase. Notably, like amcasertib exhibited the ability to inhibit invasion and migration in MDAH-2774 and OCSC cells, when we compared the effects of amcasertib to the control group, we found that it greatly reduced invasion and migration in BCSCs (23). CSCs exhibit an EMT profile, involving enhanced invasive properties and migratory capacity, and resistance to apoptosis (45,46). Reportedly, Oct-4 and Nanog increased the EMT of BCSCs, which was linked to a poor prognosis in breast cancer patients (47). Studies report that in drug-resistant MDA-MB-231 cells constructed, the EMT profile is reduced, and drug resistance is also overcome, in correlation with inhibition of stemness markers (Nanog, OCT4, SOX2, and Kif4) (37,48). Also, by validating with experiments of silencing of Nanog, amcasertib was indicated to inhibit Nanog and CD133 expression, and so to increase the growth-inhibitor effect of gefitinib in PC-9/GR cells (22). Thus, we conclude that amcasertib decreased the EMT profile of BCSCs via inhibiting CD133, Nanog, and stemness-kinases, we would like to emphasize its potential ability for drug resistance to overcome.

5. CONCLUSION

We determined that amcasertib suppressed the EMT of BCSCs acting on breast cancer recurrence and drug resistance. We suggest that amcasertib is a potential agent in the therapy of patients with breast cancer, particularly in overcoming drug resistance and recurrence.

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Conflicts of interest: *The authors declare that they have no known conflict of interests such financial or personal relationships that could have appeared to influence the work reported in this paper.*

Ethics Committee Approval: *This study doesn't require ethical approval because of the use of a commercial cell line. We submitted the Declaration Form That The Article Does Not Require Ethics Committee Permission.*

Peer-review: *Externally peer-reviewed.*

Author Contributions:

Research idea: AA, HGK, NPÖ
Design of the study: AA, HGK, NPÖ
Acquisition of data for the study: AA, HGK, NPÖ
Analysis of data for the study: AA, HGK, NPÖ, CG
Interpretation of data for the study: AA, HGK, NPÖ
Drafting the manuscript: AA, HGK, NPÖ
Revising it critically for important intellectual content: CG
Final approval of the version to be published: AA, HGK, NPÖ, CG

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