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Effects of EGFR inhibitor AG 1478 on MDA-MB-231 and MCF-7 breast cancer cells

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Abstract

In this current study, antiproliferative effect of EGFR inhibitor AG1478 was investigated in human breast cancer cell lines. MDA-MB-231 and MCF-7 cell lines were used respectively as triple negative breast cancer and Luminal A breast cancer model. To this end cell viability, cell index values by xCELLigence Real-Time Cell Analysis DP instrument and mitotic index analysis were used. The results of the current study showed that AG1478 had cytostatic effects on both of cell lines. The IC50 concentration was determined as 50 µM for MDA-MB-231 and 20 µM for MCF-7 cell line. IC50 concentration was used for mitotic index parameter. IC50 concentrations decreased the mitotic index values of both of cell lines. There were significant differences between the control and the experimental groups ($p<0.05$). The results of the present study suggest that AG1478 may serve as a promising treatment option for breast cancer.

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1. Introduction

Breast is a bilateral organ which is diagnosed with the highest number of malignancies in women and has major changes in size, shape and function during adolescence, pregnancy, breastfeeding, and postmenopausal periods [1]. It is a heterogeneous disease with various risk factors and clinical features. Although it is generally known as female disease, it is reported that researches can also develop in men [2]. Incidence rate increases with age up to 45-50 years. Lifetime estrogen exposure is one of the most important risk factors. Early menarche, late menopause, late and a small number of pregnancies, postmenopausal hormone replacement therapy, xenoestrogens and childlessness prolong the exposure to estrogen [3]. On the other hand, age, family history, the use of oral contraceptives, radiation exposure, alcohol use and benign breast disease are among the risk factors for breast cancer [4]. The prolonged exposure to estrogen and the prolongation of proliferation times increase the number of cells that can be mutated and start to proliferation of the cells that begin to tumour. Signals that cause differentiation of breast ductal cells, especially during pregnancy and other strong signals cause intense apoptosis of the alveolar and ductal cells after weaning. These cycles may be involved in the elimination of tumor cells and purification of tissue. Therefore, it is thought that many pregnancies at an early age can have a strong protective effect with long periods of breastfeeding [5, 6].

BRCA genes encode various proteins involved in the DNA repair mechanism. The germline mutations in BRCA genes are associated with breast cancer. In the examined populations, mutations on the BRCA-1 and BRCA-2 genes caused a high increase in the risk of breast cancer. However, the incidence of mutations in the BRCA-2 gene is higher than in the BRCA-1 gene [7]. BRCA1 and BRCA2 have different functions. While the protein encoded by the BRCA 1 gene is involved in estrogen receptor signaling pathway, the BRCA2 gene is involved in terminal differentiation of mammary epithelial cells [8, 9].

Epidermal Growth Factor Receptor (EGFR), a transmembrane protein, binds to peptide growth factors of the Epidermal Growth Factor (EGF) family to activate [10, 11]. EGF binds to EGFR to stimulate cell growth, proliferation and differentiation. EGFR overexpression gives tumors an aggressive phenotype and it is common in many types of solid tumors [12]. EGF and EGFR are involved in many aspects of the

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development of carcinomas [13, 14]. Studies have shown that overexpression of the EGF receptor plays an important role in pathological processes such as tumorigenesis and progression. Overexpression of EGFR is associated with poor prognosis and reduced survival, especially in different types of carcinomas [15-17].

In recent years, EGFR has an important role in cancer treatment studies. Tyrosine kinase inhibitors and monoclonal antibodies are molecules that target EGFR. It binds competitively to the ATP pocket of EGFR to inhibit tyrosine kinase activity. In contrast, antibodies competitively inhibit ligand binding to EGFR and thus inhibit receptor activation [18]. The majority of protein tyrosine kinase inhibitors disrupt signal transduction by binding the enzyme to the ATP binding pocket and are currently used in clinical trials [19]. AG 1478 is a specific epidermal growth factor receptor tyrosine kinase inhibitor used in laboratory studies [20]. In recent years, targeted treatment approaches have replaced classic methods in breast cancer, as in many cancers [21].

In this study, it was aimed to investigate the antiproliferative effects of the EGFR inhibitor AG1478 on triple negative and luminal A breast cancer cell lines.

2. Materials and Methods

2.1. Cell culture

MDA-MB-231 cells were cultured in DMEM (high glucose) (Sigma) containing 10% fetal bovine serum (Sigma), 100 µg/ml streptomycin (Ulugay), 100 IU/ml penicillin (Pfizer), amphotericin B (Sigma) at 37ºC in humidified atmosphere of 5% CO_2. TMCF-7 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Sigma), 100 µg/ml streptomycin (Ulugay), 100 IU/ml penicillin (Pfizer), amphotericin B (Sigma). 37ºC in an atmosphere humidified with 5% CO_2 for both cell types. The pH of the medium was adjusted to 7.2 with sodium bicarbonate.

2.2. Seeding of cells

In order to calculate the relative viability, seeding was made in 96-well plates with 30,000 cells / 200 µl medium per well. Sterile round coverslips were placed in each well of 24-well plates for the mitotic index parameter. Cell seeding was carried out at 150,000 cells / 300 µl medium per well. After the cells adhered to the coverslips, 500 µl of medium was added to each well. The cells were prepared for the experiment by continuing the incubation in a mixture of 95% air and 5% CO_2 at 37ºC for 24 hours.

2.3. Preparation of inhibitor concentrations

The experiments were carried out at different concentrations of EGRF inhibitor AG 1478 and at different time intervals. AG 1478 hydrochloride (Tocris) is stored as 10 mg powder at -20ºC. AG 1478 was dissolved with DMSO to obtain a 10mM stock solution and stored at + 4ºC during the experiments. Concentrations to be applied to cells in experiments were obtained by diluting the stock solution with tissue culture medium.

2.4. Cell viability (MTT)

The cytotoxicity of AG 1478 on the cells as a result of the application of different doses was evaluated with the MTT test. Different concentrations of AG 1478 were applied to the cells. At the end of the experiment periods, the medium from the wells was removed and 40 µl MTT (5mg / ml) was added. After waiting for 4 hours, 160 µl DMSO was added to the wells with MTT and left for 1 hour incubation in shaker. After the dissolution of the formed formazan crystals by this process, the absorbance values of the experimental groups were measured by spectrophotometer at 570 nm by taking the 690 nm wavelength as reference.

2.5. xCELLigence real-time cell analysis (RTCA): cytotoxicity

The basic principle of the xCELLigence RTCA-DP system is that as the amount of cells adhering to the surface of the gold-coated E-Plate increases, the resistance against current increases and as the amount of cells adhered to the surface decreases, its resistance decreases. Cell proliferation and cell death can be recorded continuously and in real time through the xCELLingence RTCA-DP system.

16-well E-Plate was used to evaluate the cell index parameter. Later, 5,000 cells in 100 µl medium for MDA-MB-231 cells and 10,000 cells in 100 µl medium for MCF-7 cells were seeded in each well. After seeding process, E-Plates were incubated for 20 minutes in a sterile working cabinet at room temperature and then placed in xCELLigence DP device and incubation continued at 37ºC and 5% CO_2 ambient conditions. After cell seeding was performed on the E-Plates, the xCELLigence DP device was commanded to take measurements every 15 minutes. Approximately 24 hours after cell seeding, in 1/3 of the proliferation phase of the cells, the medium in the E-plates was replaced with the medium containing inhibitors, and measurements were taken at 15 minutes intervals for 72 hours. Graphs of concentration and

time dependent cell index values were transferred to the computer screen.

2.6. Mitotic index (MI)

The preparations prepared according to the experimental groups were first hydrolyzed with 1 N HCl at room temperature for 1 minute and then with 1 N HCl at 60ºC for 10 minutes. After the hydrolysis process, Feulgen method was applied to the preparations for 1 hour. These preparations were then washed 3 times with the washing solution of the Feulgen method for 2 minutes each and air dried. After the preparations dried, they were stained with Giemsa dye for 2 minutes. Late prophase, metaphase, anaphase and telophase phases were counted in order to determine the mitosis index (MI) values of the preparations ready for counting. Since the early prophase stage is morphologically similar to the cells in the interphase group, it was evaluated together with this stage. The applied inhibitor concentration was evaluated as three preparations for each time and the MI values were determined by counting an average of 3,000 cells from each preparation.

2.7. Statistical evaluation

Concentrations applied to all experimental groups and values of cell kinetics parameters determined according to time were evaluated relative to control groups and each other. One-way ANOVA test was applied to the values determined from the experimental groups. While the significance of the groups with respect to the control was evaluated with the DUNNETT's test, the significance of the groups with each other was evaluated with the t-test. Statistical evaluations were based on $p < 0.05$ significance level.

3. Results and Discussion

3.1. Determination of optimal concentration with cell viability analysis

In order to determine the changes caused by AG 1478 in the mitochondrial dehydrogenase enzyme activity of MDA-MB-231 and MCF-7 cell lines, initially 50 µM, 100 µM, 150 µM inhibitor concentrations were used in the cultured cells for 24 hours. The absorbance values obtained from the experimental series conducted in parallel with the control group without inhibitor applied were shown in Tables 1 and 2.

Table 1. Absorbance values of mitochondrial dehydrogenase activity of MDA-MB-231 cells treated with AG 1478 at concentrations of 50 µM, 100 µM ve 150 µM for 24 h ($p<0.05$).

Experimental Groups	Absorbance Values (450-690 nm)
Control	$561,636 \times 10^{-3} \pm 0,014$ SD
50 µM	$289,5 \times 10^{-3} \pm 0,011$ *
100 µM	$266,15 \times 10^{-3} \pm 0,009$ *
150 µM	$184,1 \times 10^{-3} \pm 0,008$ *

Table 2. Absorbance values of mitochondrial dehydrogenase activity of MCF-7 cells treated with AG 1478 at concentrations of 20 µM, 100 µM ve 150 µM for 24 h ($p<0.05$).

Experimental Groups	Absorbance Values (450-690 nm)
Control	$561,636 \times 10^{-3} \pm 0,014$ SD
20 µM	$288,166 \times 10^{-3} \pm 0,012$ *
100 µM	$180 \times 10^{-3} \pm 0,008$ *
150 µM	$143,166 \times 10^{-3} \pm 0,007$*

When the absorbance values are examined viability values were 51,8% for 50 µM; 43,63 for 100 µM and 32,95 for 150 µM compared to control group which was considered as 100% for MDA-MB-231 cell. For MCF-7 cell line these values were 51,76% for 20 µM; 45,42 for 100 µM and 37,94 for 150 µM (Figure 1 and 2).

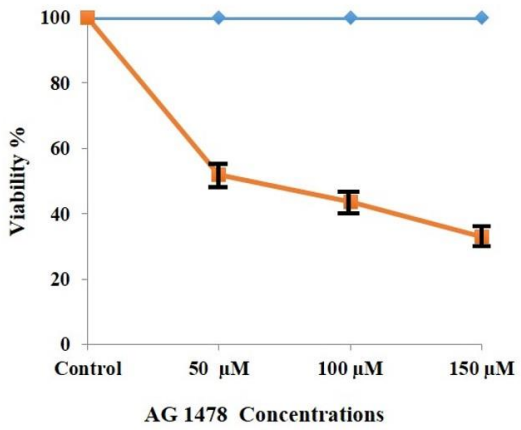

Figure 1. Percent viability values of MDA-MB-231 cells treated with 50 µM, 100 µM, 150 µM AG 1478 for 24 h ($p<0.05$).

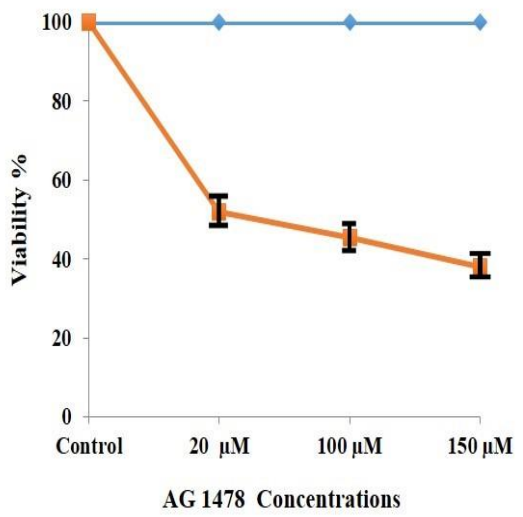

Figure 2. Percent viability values of MCF-7 cells treated with 50 µM, 100 µM, 150 µM AG 1478 for 24 h ($p<0.05$).

According to the data obtained, it is seen that 50 µM AG 1478 for the MDA-MB-231 cell line and 20 µM AG 1478 for the MCF-7 cell line were the IC50 concentrations that cause the death of half of the cells.

3.2. xCELLigence Real-Time Cell Analysis (RTCA): cytotoxicity

When the cell index values obtained from the real-time cell analysis system as a result of the application of AG 1478 to the MDA-MB-231 cell line at concentrations of 50 µM, 100 µM and 150 µM to the MCF-7 cell line at 20 µM, 100 µM and 150 µM concentrations were examined, inhibitor appeared to have antiproliferative effects on cells. The curves of the graph of the cell index values, when compared with the standard curves, suggest that all the applied AG 1478 concentrations produced a cytostatic effect in both cell lines (Figure 3 and 4).

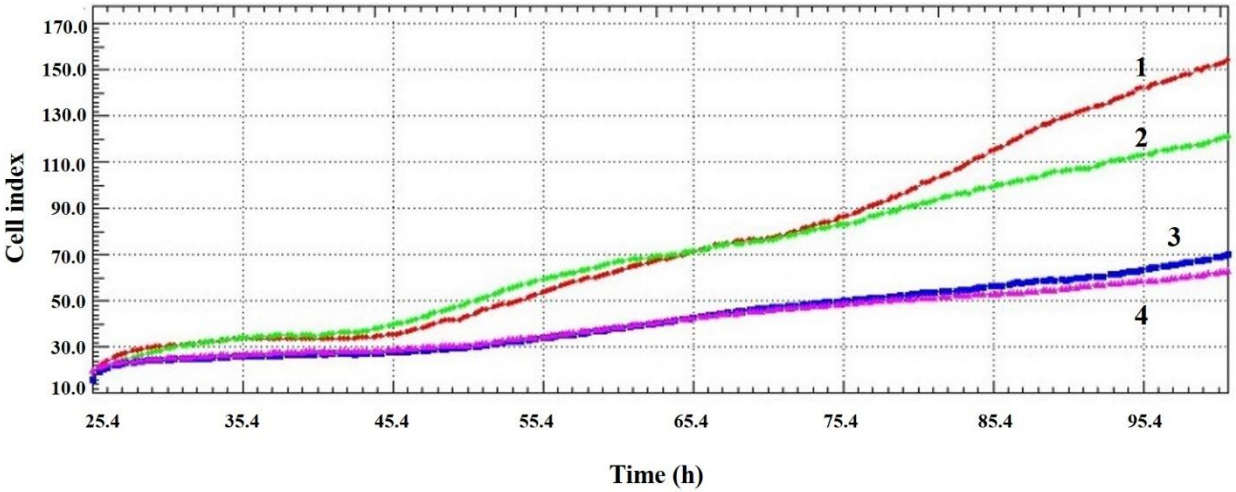

Figure 3. Graph of cell index of MDA-MB-231 cells treated with AG 1478 at concentrations of 50 µM, 100 µM ve 150 µM (Line 1: Control, Line 2: 50 µM, Line 3: 100 µM , Line 4: 150 µM).

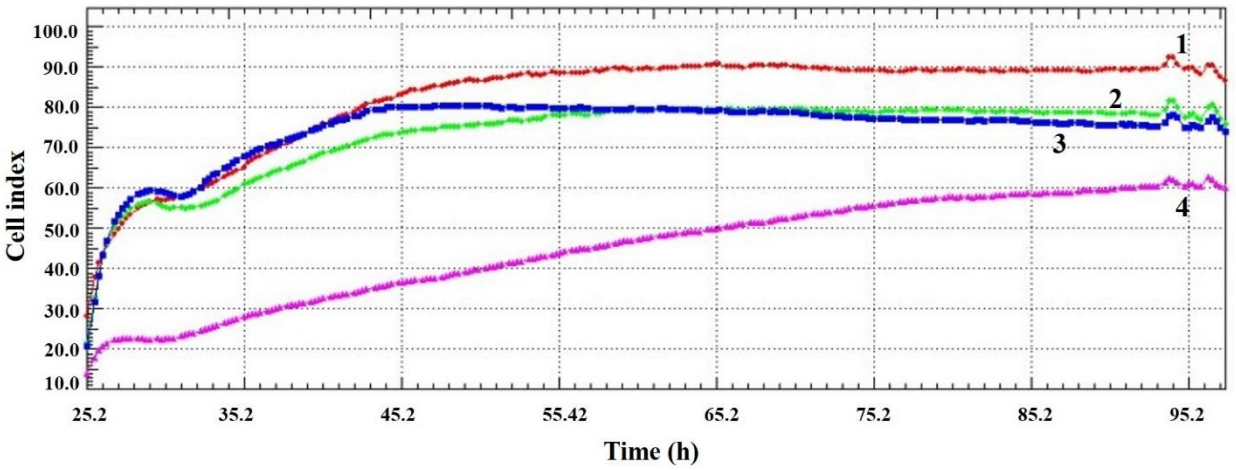

Figure 4. Graph of cell index of MCF-7 cells treated with AG 1478 at concentrations of 20 µM, 100 µM ve 150 µM (Line 1: Control, Line 2: 20 µM, Line 3: 100 µM , Line 4: 150 µM).

3.4. Mitotic index (MI)

In order to determine the change in mitotic index values as a result of the application of AG 1478 on cells; inhibitor concentrations of 50 µM were applied to MDA-MB-231 cells and 20 µM to MCF-7 cells, which were cultured for 0-72 hours. The mitotic index values obtained as a result of the experiment conducted in parallel with the control group without inhibitors are shown in Table 3 and Table 4. As seen in Figure 5 and Figure 6, mitotic index values of MDA-MB-231 and MCF-7 cells decreased significantly depending on time as a result of AG 1478 application.

Table 3. Mitotic index valuees of MDA-MB-231 cells treated with AG 1478 at concentrations of 50 µM for 0-72 h ($p<0.05$).

Time (h)	Mitotic Index (%)	
	Control	50 µM
24	6,13±0,03^{SD}	2,6±0,02*
48	6,6±0,04	0,93±0,01*
72	6,7±0,03	0,34±0,01*

Table 4. Mitotic index valuees of MCF-7 cells treated with AG 1478 at concentrations of 20 µM for 0-72 h ($p<0.05$).

Time (h)	Mitotic Index (%)	
	Control	50 µM
24	4,7 ± 0,02^{SD}	2,08 ± 0,02*
48	5,8 ± 0,03	2,06 ± 0,01*
72	5,4 ± 0,03	1,8 ± 0,01*

4. Discussion

Developing the treatment methods of cancer, one of the most important diseases of our age, directing the treatments to specific targets in line with the purposes of increasing the effectiveness of the methods used and eliminating the side effects increases the survival chances of the patients.

Triple negative breast cancer has a poor prognosis and an aggressive phenotype. It is insensitive to drugs that target hormone receptors and human epidermal growth factor receptor 2. Therefore, the development of an effective therapeutic reagent to treat triple negative breast cancer is required [22].

Bishop et al. observed that in their studies examining the sensitivity of cancer cells against different inhibitors of the EGFR family, it was observed that HER1 and MAPK signaling in all HER1-expressing cells was inhibited by AG 1478. In this study, in cell lines with high HER1 expression and where AG 1478 mediates the arrest of cells in the G1 phase. It has been observed that at low concentrations the inhibitor potentially inhibits both HER1 phosphorylation and the MAPK pathway, whereas cell lines expressing low HER1 require higher inhibitor concentrations for the same effects [23].

In this study, different concentrations of AG 1478 were applied to triple negative and Luminal A breast cancer cell lines and evaluated with xCELLigence Real Time Cell Analysis System. As a result of the comparison of the obtained cell index values and the curves obtained from these values with the standard curves, it is suggested that the AG 1478 have a cell growth inhibitory effect for both cell lines. This situation is in accordance with the fact that AG 1478, mentioned in the above literature, mediates the pause in the G1 phase.

In Zhang et al.'s study on MCF-7 and MDA-MB-231 cell lines have been shown that AG1478 was able to inhibit the activation of EGFR, ERK1 / 2 and AKT signaling pathways, and although the two cell lines expressed EGFR at different levels, the inhibitor had similar antiproliferative activity on cell lines [24].

Studies with AG 1478 show that this inhibitor has antitumor activity. In *in vitro* studies, the inhibitor showed significant antiproliferative effects on glioblastoma, leiomyoma, colorectal carcinoma and nasopharyngeal carcinoma cells [25-28]. It has also been shown to sensitize tumors to the cytotoxic effect of cisplatin and temozolomide or to the monoclonal antibody mAb 806, which is an anti-EGFR antibody [29-31].

The use of EGFR/HER1 inhibitor AG 1478 with HER2 inhibitor has been shown to synergistically reduce cell viability in breast cancer [32]. It has been shown that the use of tamoxifen with AG 1478 in breast cancer patients inactivates the EMT program that plays a role in the metastasis process [33]. Treatments of the EGFR inhibitor AG1478 in SkBr3 cells have been shown to abolish the cytochrome P450 1B1 (CYP1B1) expression [34].

In recent years, many studies have found that estrogen and estrogen receptors play critical roles on breast tumors. ER and / or progesterone receptors are expressed in approximately 70% of breast tumors [35]. Tsonis et al. showed that the coordinated action of ERs with EGFR and / or IGFR in the expression of bioactive ECM macromolecules that play a role in cancer progression is very important and powerful agents for endocrine therapies [36].

In this study; The effect of EGFR inhibitor AG 1478 on MDA-MB-231 as a triple negative breast cancer model and MCF-7 cell lines used as a Luminal A breast cancer model was evaluated using cell kinetics parameters such as cell viability, cell index, and mitotic index. The data obtained as a result of the study show that there is a significant decrease in cell viability, cell index, mitotic index values depending on time.

These data suggest that treatments for EGFR in breast cancer and other cancer types will have positive results in cancer treatment. The findings show that treatment strategies can be developed by targeting HER1 in HER2-negative cancer types that are difficult to treat, such as triple negative breast cancer. It is also thought to be useful in the treatment of treatment-resistant cancers.

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Conflicts of interest

The authors state that did not have conflict of interests.

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