Investigating the Cytotoxic and Anti-proliferative Effects of Trastuzumab on MDA-MB-453 and MDA-MB-468 Breast Cell Lines With Different Levels of HER2 Expression

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Abstract

Introduction: Trastuzumab is a common treatment for HER2-positive breast cancer. Trastuzumab exerts its effect through inhibiting intracellular signaling pathway induced by HER2. This study aimed to specifically investigate the cytotoxic effect of trastuzumab against two different breast cell lines, MDA-MB-453 (HER2-high) and MDA-MB-468 (HER2-low).

Materials and Methods: The breast cancer cell lines were subjected to various concentrations of trastuzumab (1-1000 ng/mL). The trastuzumab’s effects were regularly monitored via direct observation by inverted microscopy. Effects of trastuzumab were determined on cytotoxicity, cell proliferation and apoptosis at 24 and 72 hours post-treatment via MTT colorimetric, cell cycle and apoptosis assays.

Results: Microscopic observation demonstrated a dose-dependent increase in cell death at treated ones. The MTT assay showed that trastuzumab (1-1000 ng/mL) inhibited the growth of both cell lines in a dose-dependent manner. The findings of the present study revealed that trastuzumab induces a statistically higher cytotoxicity at all concentrations in MDA-MB-453 compared to MDA-MB-468 cells. It has been actually revealed that trastuzumab suppresses cell proliferation through inducing G1 phase arrest and triggers apoptosis in both cell lines. However, the effect of trastuzumab was found to be higher in MDA-MB-453, compared to MDA-MB-468.

Conclusions: Trastuzumab could inhibit cell proliferation and trigger apoptosis in HER2-positive cells. Although Trastuzumab affected both cell lines, it significantly inhibited the cell growth of HER2-high cells.

Keywords: Trastuzumab, MDA-MB-453, Breast Cancer, MDA-MB-468, Cytotoxicity, Apoptosis


Introduction

Breast cancer is considered to be a prevalent malignant neoplasm in females, accounting for the second highest lethal diseases in all types of cancers worldwide. Surgery, radiotherapy, hormone therapy, chemotherapy, as well as targeted drug delivery are the current therapeutic options for treating breast cancer patients.¹ ³ Over the past decade, the human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells have been subjected to studies regarding discoveries or improvements in cancer therapy strategies.⁴

The HER2 is a member of the epidermal growth factor receptor (EGFR) family of transmembrane receptor tyrosine kinases, triggering the downstream signaling via the phosphoinositide 3-kinase/AKT and MAPK pathways.⁵ ⁷ Activation of this signal leads to growth, migration, proliferation, survival, and angiogenesis.⁸ A wide variety of in vitro and in vivo studies have demonstrated a relationship between high levels of HER2 and early stage breast malignancy, cancer metastasis, and the diminished efficiency of hormone therapy.⁹ Importantly, the overexpression of the HER2 receptor tyrosine kinase is found to be associated with a poor survival.¹⁰

Application of monoclonal antibodies (mAbs) for molecular targeted therapy is one of the successful methods for treating cancer patients. Trastuzumab (Herceptin®), is a recombinant humanized mAb, capable of binding to HER2 which has been approved to treat patients with HER2-amplified breast cancer. When Trastuzumab is combined with chemotherapy, it could provide better outcomes in improved progression-free survival among HER2-positive patients.¹¹ ¹²

The most controversial mechanism of Trastuzumab is that it exerts its cytotoxicity effects at inhibiting HER2 activation. The main reason underlying this argument is various cellular backgrounds of different breast cancer cells applied in a variety of investigations. Each breast cell line exhibits an exceptional expression signature of different HER receptors, which can
considerably influence the efficacy of Trastuzumab.\textsuperscript{13}

To resolve this issue, this research was devoted to investigate the anticancer effects of Trastuzumab in two various cell types of breast cancer with different levels of HER-2 expression via in vitro experiments. This study intended to specifically examine the sensitivity of MDA-MB-453 and MDA-MB-468 cells (as HER2-high and -low, respectively) to this antibody.

Materials and Methods

Cell Lines and Reagents

The MDA-MB-453 and MDA-MB-468 (breast cancer cell lines) were bought from the National Cell Bank of Iran (NCBI; Pasteur Institute of Iran) cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich) complemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 unit/mL penicillin, 100 μg/mL streptomycin and 0.2 mM Glotamax (Invitrogen Gibco), at 37°C with 5% CO\textsubscript{2} and 95% humidity. Detaching the cells were carried out by trypsin-EDTA (Sigma, St Louis, MO) at nearly 80% confluency, enumerated by Neubauer hemocytometer. The cells were incubated for 24 and 72 hours upon treatment.

Preparation of Trastuzumab

Trastuzumab (brand name: Herceptin\textsuperscript{\textregistered}) was provided from the Roche company (Switzerland). Trastuzumab was stored at -20°C in a lyophilized form. Freshly-prepared stock solution was used for all the experiments, through dissolving Trastuzumab, in the lyophilized protein, in the medium. The solutions of Trastuzumab were purified and concentrated on Amicon Centricon Filters (Millipore; Billerica, USA) with 30-kDa molecular weight cutoff and stored in sterile (frozen aliquots). The concentration and purity of Trastuzumab were confirmed using UV absorption at 280 nm and SDS-PAGE, respectively.\textsuperscript{14}

Cell Viability Assay by Trypan Blue Dye Exclusion

Cell suspensions were gently mixed with equal volumes of 0.4% trypan blue reagent. The solution was then completely mixed and the numbers of viable and nonviable cells were confirmed by trypan blue and hemocytometer. The viability was enumerated in the formula as follows: Viability\% = (living cell count/total cell) × 100

Cellular Morphology

The effects of Trastuzumab on cell morphologies were investigated by inverted light microscopy (Leica, Inc.) at 0, 24 and 72 hours upon treatment.

Colorimetric Cytotoxicity Assay

The in vitro cytotoxic activity was determined by MTT assay. Cell suspensions containing 12 × 10\textsuperscript{4} viable MDA-MB-453 and MDA-MB-468 were seeded in a 96-well tissue cultivating plate (Greiner, Frickenhausen, Germany). Cells with 80% confluency were exposed to Trastuzumab at various concentrations, including 1, 10, 100, 500, and 1000 ng/mL. Cells without treatment were considered as a negative control. Following the incubation of 24 and 72 hours, the media was discarded, cells were washed by PBS twice, and 100 μL/well of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, 5 mg/mL in PBS) was supplemented. Afterwards, cells were maintained at 37°C for 4 hours. After the incubation, medium was discarded, and formed formazan crystals in the cells were dissolved at 200 μL of dimethyl sulphoxide (DMSO, Sigma Aldrich, USA). Afterwards, plates were shaken for 1 hour at 37°C. Finally, the absorbance was analyzed by microplate readers at the 570-nm wavelength. The absorbance of cells with no treatment was determined as 100% cell survival. The cytotoxicity levels were determined by the formula as follows:

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\text{Cell cytotoxicity (\%)} = 100 - \left(\frac{\text{Absorbance value of the test compound} - \text{Absorbance value of the blank}}{\text{Absorbance value of the negative control} - \text{Absorbance value of the blank}}\right) \times 100
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Cell Cycle Analysis

Cell lines were cultured at 5 × 10\textsuperscript{5} cell in 12-well plate for 24 hours, separately. One day upon seeding, the cells were subjected to 500 ng/mL Trastuzumab (test group) and untreated cells (control group) were incubated for 72 hours. Afterwards, both cell lines were harvested and washed with cold PBS twice. Then, they were resuspended and fixed overnight in 70% ice-cold ethanol and were stored at 4°C. After washing cells twice with PBS, the cells were sedimented by centrifugation, then suspended in 0.5 mL RNase A (100 μg/mL)-containing PBS and incubated for 30 minutes at 37°C. Subsequently, the cells were dyed with 50 μg/mL propidium iodide (100 μg/mL) on ice in PBS for 1 hour in the dark. The distribution of the cell cycle (the percent of cells at the different phases G0/G1, S, and G2/M) as well as the percent of cells at sub-G1 DNA peak were evaluated via flow cytometry.

Analyzing Annexin V/PI Apoptosis

Apoptosis analysis was done using fluorescein isothiocyanate (FITC)-labeled Annexin V/propidium iodide (PI) staining based on the manufacturer’s protocol. A density of 5 × 10\textsuperscript{4} MDA-MB-453 and MDA-MB-468 cells were cultivated in a 12-wells plate, and were attached for 24 hours. Test group cells were treated with 500 ng/mL of Trastuzumab and untreated cells were considered as the control group for 72 hours. Upon incubation, the adherent cells were trypsinized, harvested, counted, washed twice with PBS containing 2% FBS and suspended in Annexin V binding buffer. Afterwards, cells were transported in 5 mL test tubes (in 1 mL of PBS containing BSA), followed by fixation through supplementing 5 μL of Annexin V-FITC and 10 μL of PI solution. Cells were then gently vortexed and maintained in the dark for 15 minutes at RT. Subsequently, the stained cells were directly examined via flow cytometry. Data analysis was conducted by FlowJo v.7 software.

Statistical Analysis

Statistical analysis and graphical illustration of data were performed by GraphPad Prism (GraphPad Prism, version 6.00). Each test was performed at least with three times repeat. All the data have been represented as mean ± standard deviation (SD) of three independent experiments. One-way
ANOVA was used for determining the statistical significance for multiple (>2) groups. The $P$ values less than 0.05 were regarded statistically meaningful; $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***), and $P<0.0001$ (****).

**Results**

**Microscopic Evaluation of Trastuzumab on Cell Cytotoxicity**

The cells were treated for 24 hours with Trastuzumab at concentrations ranging from 1, 10, 100, 500, and 1000 ng/mL. Untreated cells were considered as negative controls. Before and after Trastuzumab treatment, cell viability was routinely monitored by an invert microscope. As shown in Figure 1, microscopic observations revealed the ability of Trastuzumab to trigger cell death specifically at concentrations of 500 and 1000 ng/mL.

**Studying the Effect of Trastuzumab on Cytotoxicity by MTT Colorimetric Assay**

To investigate the cytotoxicity effect of Trastuzumab, cell lines were exposed to different concentrations (1, 10, 100, 500, and 1000 ng/mL) of Trastuzumab. The results provided by the MTT assay revealed that the cytotoxicity outcome of Trastuzumab on MDA-MB-453 and MDA-MB-468 cell proliferation has a dose- and time-dependent pattern (Figure 2).

As shown in Figure 2, Trastuzumab at different concentrations ranging from 1 to 1000 ng/mL had a significant cytotoxicity on MDA-MB-453 cells, compared to Trastuzumab-treated MDA-MB-468 as well as cells with no treatment. Trastuzumab exhibited a significant increase in cell death after 24 and 72 hours at a concentration of 1 to 1000 ng/mL in MDA-MB-468 cells compared to Trastuzumab-treated MDA-MB-453. It was revealed that MDA-MB-453 had a significantly increased cell death rate at all concentrations compared to MDA-MB-468. In addition, lower concentrations of Trastuzumab showed more increased cell death in MDA-MB-453 compared to MDA-MB-468 (Figure 2). This test indicated that Trastuzumab has less significant effects on MDA-MB-468 as a HER2-low cell line. Additionally, concentrations of 500 and 1000 ng/mL showed significance differences with other doses such as 1, 10, and 100 ng/mL in both MDA-MB-453 and MDA-MB-468 cell lines.

**Analysis of Cell Cycle Arrest after Trastuzumab Treatment**

To determine the cell cycle arrest in the Trastuzumab-induced cytotoxicity, distribution and progression of cell cycle level was determined in MDA-MB-453 and MDA-MB-468 exposed to 500 ng/mL of Trastuzumab using flow cytometry following PI staining. This study demonstrated that Trastuzumab causes G1 phase cell cycle arrest in MDA-MB-453 and MDA-MB-468 cell lines (Figure 3). Generally, Trastuzumab produced lower cell percentages in G2/M and S phases in comparison to the control. Additionally, Trastuzumab induced a sub-G1 peak, suggesting the induction of apoptosis. Trastuzumab resulted in 7.55% and 3.82% of MDA-MB-453 and MDA-MB-468, respectively, in the sub-G1 DNA peak (Figure 3).

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**Figure 1.** Microscopic observation of cells treated with Trastuzumab. (A) Cells with no treatment. (B) Formation of formazan crystals in cells treated with Trastuzumab.

**Figure 2.** The Activity of Trastuzumab on MDA-MB-453 and MDA-MB-468 Cell Lines. Both cell lines were subjected to different concentrations of Trastuzumab for (A) 24 and (B) 72 hours, and cell viability was inspected using the MTT assay. Significant cell death was witnessed in Trastuzumab-treated MDA-MB-468 after 24 and 72 hours, compared with Trastuzumab-treated MDA-MB-453. Results are mean of three independent experiments ± standard error. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$.**
Investigation of Apoptosis After Trastuzumab Treatment

In order to find the role of apoptosis in the Trastuzumab-induced cell growth suppression, cells treated with 500 ng/mL of Trastuzumab were dyed with Annexin V-FITC/PI. MDA-MB-453 and MDA-MB-468 were exposed to Trastuzumab for 72 hours. A concentration of 500 ng/mL of Trastuzumab showed to have the ability of triggering apoptosis in both cell lines (Figure 4). However, a clearly increased apoptosis was detected in MDA-MB-453 cells, as compared to MDA-MB-468 cells. It was demonstrated that the Trastuzumab treatment led to remarkably induced apoptosis in comparison with the control cell group. Trastuzumab was found to block the progression of the cell cycle and selectively induced apoptosis. It actually resulted in total apoptosis as amount of 35% and 19.05% Annexin V-positive MDA-MB-453 and MDA-MB-468, respectively (Figure 4).

Discussion

Overexpression of HER2 in breast cancer tissue is related to shorter survival. Although Trastuzumab exhibits high efficacy in HER2-positive invasive breast cancer patients, there are still some patients who cannot appropriately be treated with Trastuzumab.15-17 The various cellular profiles of different breast cancer cells are a main problem in experimental models. Indeed, every breast cancer cell displays an exclusive expression background of different HER receptors, which can efficiently influence the Trastuzumab activity. In the present study, it was intended to examine the sensitivity of
HER2 Expression Level and Trastuzumab Efficacy

cell lines with different HER2 background. Accordingly, MDA-MB-453 and MDA-MB-468, as HER2-high and -low cells, respectively, were exposed to various concentrations of Trastuzumab and cell cytotoxicity was measured in a dose-dependent fashion using MTT, cell cycle, and apoptosis assays. The results provided by this study shed light on the potent cytotoxic activity of Trastuzumab on human cancer cell line MDA-MB-453, compared to MDA-MB-468 and the control. Regarding the expression profile of HER-2, MDA-MB-453 cells were determined as 2+,

representing a minimum HER-2 profile expressed by breast cancer for which Trastuzumab is clinically used.

This study demonstrated that the efficacy of Trastuzumab is directly linked with the expression profile of investigated cell lines. These findings are in agreement with the findings of Merlin et al who demonstrated the direct relationship of Trastuzumab cytotoxicity with HER2 expression level. In another study, Ginestier et al reported that Trastuzumab's sensitivity is commonly relied on the ability of expressing phosphorylated ERBB2.

Effects of Trastuzumab on the cell lines showed better outcomes after 24 hour exposure compared with the 72-hour exposure. This can be clarified because Trastuzumab may have the best activity in the first 24 hour treatment. In addition, the lower concentrations of Trastuzumab showed better results in both 24 and 72 hours' exposure. Although being a cytostatic agent with the growth factor receptor binding ability and preventing cell growth pathway mediated by HER-2, Trastuzumab could trigger cell growth via other pathways. This may explain why increased concentrations of Trastuzumab did not make obvious differences on cell cytotoxicity.

In addition, the influences of Trastuzumab on the cell cycle in cell lines were analyzed. The dysregulation of the cell cycle is considered as a marked hallmark of cancer, presumably suggesting cell cycle arrest induction as an attractive and efficient method to treat abnormal cancer cell growth. The results of cell cycle arrest assay by this study confirmed that Trastuzumab inhibits MDA-MB-453 and MDA-MB-468 growth via G1 phase arrest. In Trastuzumab-exposed MDA-MB-453 cells, cell cycle arrest was induced at the G1 phase (Figure 3, top right), in which cell population present in the G1 phase increased significantly when compared to Trastuzumab-un-treated MDA-MB-453 cells (Figure 3, top left). Interestingly, in Trastuzumab-treated MDA-MB-468 cells, cell population increased significantly in the G1 phase (Figure 3, bottom right) compared to Trastuzumab-un-treated MDA-MB-468 cells (Figure 3, bottom left). Actually, in both cancer cells, Trastuzumab increased the number of cells at the G1 phase. In addition, in both tested breast cancer cell lines, Trastuzumab decreased the cell percentage in S and G2/M phases, specifically in MDA-MB-453 cells. In contrast, Trastuzumab increased fractions of cells in the sub-G1 phase in both cells tested, specifically in MDA-MB-453 cells, proposing apoptosis induction.

In spite of these findings, a study conducted by Chen et al in 2011 demonstrated that the treatment of the SKBR3 cells, HER2-positive breast cancer cell line, by Trastuzumab could not arrest cell cycle and induce apoptosis; however, a combination of Trastuzumab and Sodium butyrate (NaB) caused an obvious enhancement in the induction of both cell cycle arrest and apoptosis. Nevertheless, there is a previously-published report showing that Trastuzumab-treated cells with certain concentrations for a sufficient time can slow down the cell cycle progress and trigger cells into dormancy through increased expressing HEC and DEEPEST genes, mostly in the G2/M phase.

Collectively, such finding proposed that Trastuzumab might change expression levels of cell-cycle associated proteins to trigger G1 phase halting and subsequently preventing proliferation of MDA-MB-453 and MDA-MB-468.

Apoptosis is a preserved cell suicide mechanism for the elimination of damaged, redundant, or infected cells. This process can be initiated by compounds that target both extrinsic and intrinsic apoptotic pathways. The former includes interaction of ligands and their cognate cell surface death receptors, including Fas/CD95 and TNFR1, leading to protease activation of caspase-3, caspase-9, and other downstream caspases as well as the induction of the apoptotic cascade. On the other hand, the mitochondria-induced intrinsic pathway might lead to apoptosis. Findings from this study revealed that Trastuzumab has the ability to induce apoptosis in both cell lines.

The HER-2, while not overexpressed in normal tissues, is overexpressed in both primary and metastatic cancer cells, making it an appropriate candidate for targeted therapy. Nearly 20% to 30% of the patients with new diagnosis of invasive breast malignancy show HER2 protein overexpression or gene amplification, causing higher mortality rates, elevated metastasis incidence, reduced relapse time, and unfavorable prognosis. In vitro and in vivo investigations demonstrated that HER-2 inhibition through antibodies against HER2 leads to increased cytotoxicity in breast cancer cells. The results from clinical trials has shown that Trastuzumab has a 21% efficacy in metastatic breast cancer patients. However, the Trastuzumab-resistant profile has been reported in many patients. Crawford et al showed that ABT-737 and Trastuzumab combination results in reduced proliferation of cells with Trastuzumab resistance more significantly than either drugs alone.

Conclusions
The results presented by the current study indicates that Trastuzumab has unique biological and molecular effects on MDA-MB-453 and MDA-MB-468 cells, reducing cellular proliferation and inducing cytotoxicity and apoptosis via the induction of specific and selective molecular effects. However, the different expression of HER-2 in breast cancer cell lines influences the cytotoxicity potential of Trastuzumab. Therefore, Trastuzumab, alone or combined with different anti-cancer compounds, has to be applied regarding the expression profile of HER-2. This proposes that the expression levels of HER2 could be used for choosing the most appropriate drug in combining Trastuzumab with other chemotherapy drugs for breast cancer.
Authors’ Contributions
All authors contributed equally to this study.

Conflict of Interest Disclosures
The authors declare they have no conflicts of interest.

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