Inhibition of Erbb2 by Trastuzumab Induces Oxidative Stress Markers in HER2 Positive Breast Cancer Cell Lines

Zahra Mohammadi Abgarmi1, Abbas Sahebghadam Lotfi1*, Saeid Abroun2, Masoud Soleimani2, Shahla Moahammad Ganji3

1Department of Clinical Biochemistry, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran
2Department of Hematology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran
3National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

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ABSTRACT

Human epidermal growth factor receptor2 (HER2) amplification occurs in approximately 30% of breast cancers that associated with a faster rate of growth and a poorer prognosis. Trastuzumab is FDA-approved humanized monoclonal antibody that targets the extracellular domain of HER2. In present study we blocked HER2 signaling pathways with trastuzumab and assessed oxidative stress markers in breast cancer cell lines. After MTT test, IC50 of trastuzumab on breast cancer cells were calculated with Compusyn software. Cells treated with various concentration of trastuzumab. Intracellular ROS determined with DCFH-DA fluorimetric probe, MDA as lipid peroxidation index detected with TBARS method. Protein carbonyl contents of cells were assessed with DNPH derivatization spectrophotometrically at 360-385 nm. Our results showed that trastuzumab treatment increases reactive oxygen species, lipid peroxidation and protein carbonyl content in HER2 positive breast cancer cell lines. These results provide evidence that increasing of oxidative stress markers in HER2 overexpressing compared to HER2 negative breast cancer cell lines after treatment with trastuzumab can be exploited for selective HER2 positive cancer cells therapy; combination of trastuzumab with ROS producing agents renders them more susceptible and induces cell death

Key words: Trastuzumab, HER2 Positive Breast Cancer, Reactive Oxygen Species, Malondialdehyde


Corresponding author: Abbas Sahebghadam Lotfi
E-mail: lotfi_ab@modares.ac.ir
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INTRODUCTION

Breast cancer is the most common malignancy among women worldwide, both in developed and developing countries [1]. Breast cancer is multifaceted disease comprised of distinct biological subtypes with diverse natural history, presenting a varied spectrum of clinical, pathologic and molecular features with different prognostic and therapeutic implications. Based on IHC profile ER/PR and HER2/neu expression, breast cancer is classified into four groups: luminal A and B (positive tumors expressing estrogen receptors-ER and progesterone receptors-PR), triple negative (also called basal-like) and HER2-overexpressed (human epidermal growth factor receptor 2, also known as ErbB2) [2].

Approximately 30% of patients with breast cancer overexpress HER2 that is correlated with gene amplification and poor clinical outcome [3]. HER2 as a ligand orphan receptor that has no ligand, forms homodimers or heterodimers with the other members of the HER family and amplifies the cellular signals [4]. Ligand-dependent activation of HER1, HER3, and HER4 by EGF or heregulin results in heterodimerization and,
thereby, HER2 activation that cause cell survival [5].

Blocking the dimerization of HER2 with other HER receptors provide a potent strategy for HER2 inhibition [6]. Trastuzumab (Herceptin) is humanized IgG1 monoclonal antibody which targets the extracellular domain of HER2 and significantly improved disease-free survival and overall survival of women with early-stage HER2-positive breast cancer [7].

Despite the clinical benefit seen with trastuzumab administration, a significant number of patients with HER2-overexpressing breast cancer will be initially or eventually resistant to anti-HER2-based therapy with trastuzumab. Understanding the mechanisms of resistance to trastuzumab is therefore crucial for the development of new therapeutic strategies [8].

ROS described as a heterogeneous group of diatomic oxygen from free and non-free radical species that are highly reactive and promote oxidative stress, which may result in cancer initiation, progression and survival phenotypes. While elevated rates of reactive oxygen species (ROS) have been detected in almost all cancers, heightened levels may serve as an indicator of drug resistance [9].

In cancer cells high levels of reactive oxygen species can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidase, cyclooxygenases, lipoxigenases and thymidine phosphorylase, or through crosstalk with infiltrating immune cells which maintains the oncogenic phenotype and drives tumor progression. Redox adaption through upregulation of anti-apoptotic and antioxidant molecules allows cancer cells to promote survival and to develop resistance to anticancer drugs [10]. Overexpression of HER2 has been associated with drug resistance in breast cancer cells [11]. Understanding relationship between ROS and HER2 may help us develop more effective cancer chemotherapeutic treatments in trastuzumab resistance breast cancer cells. In present study we aimed to block HER2 signaling pathways with trastuzumab in breast cancer cell lines and study oxidative stress markers.

**MATERIAL AND METHODS**

**Materials**
The human breast cancer cell lines (BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231 and MCF-7) were obtained from Iranian Biological Resource Centre (IBRC, Tehran, Iran). DMEM and RPMI-1640 Cell culture medium, Fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco, BRL (Lifetechnology, Paisley, Scotland). Trichloroacetic acids (TCA), phenylmethylsulphonyl fluoride (PMSF), were obtained from Sigma Chem. Co (Germany). 2′, 7′-dichloro fluorescein diacetate (DCFH-DA) was obtained from Molecular probe (Eugene, Oregon, and USA). Dimethylsulfoxide (DMSO) , thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany).

**Cell culture**
BT-474, MDA-MB-453 and MDA-MB-231 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS). SK-BR-3 and MCF-7 were maintained in Dulbecco’s minimum Eagle’s media (DMEM) with 10% FBS. BT-474 and SK-BR-3 media were supplemented with 2mM L-Glutamine. Penicillin–streptomycin (1%) was used to control bacterial contamination in all cell lines culture media. The cells cultured in 25 t flask maintained at 37°C in humidified atmosphere with 5% CO₂ and passaged every 2 days.

**MTT assay**
Cells(1×10⁴ /well) were plated in 96-well plates .for MTT assay the medium of treated and untreated wells was removed carefully and washed twice with PBS and 100 ml of MTT(5mg/ml) was added. The plate were incubated for 4 hours at 37°C with 5% CO2. 95% air and complete humidity thereafter MTT solution was removed and MTT-formazan crystals dissolved in 100 μL DMSO. Then absorbance of each plate was measured at wavelength of 570 nm and a reference wavelength of 630 nm using a plate reader.

**Determination of intracellular ROS**
Generation of ROS was detected with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorimetric nonpolar probe, which is taken up by the cells and is deacetylated by cellular esterases to polar 2′,7′-dichlorodihydrofluorescein (DCFH) that oxidized by ROS and other peroxides to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF ). Therefore the intensity of florescence is correlated with amount of ROS. For experiments stock solution of DCFH-
DA (20 mM w/v) prepared in DMSO and stored in dark at -20 °C. For determination of ROS, 2×10^4 cells per well, were seeded in 96-well plate. After overnight incubation, the cells were treated with different concentrations of trastuzumab for 48 h. After incubation time, for determination of intracellular ROS, the medium of treated and untreated wells was removed carefully and washed twice with PBS and 100 μL DCFH-DA solution (final concentration 10 μM) was added to each well and incubated at 37 °C for 45 min in dark. Fluorescence readings were taken at excitation and emission wavelengths of 480 and 530 nm respectively, in a Synergy HT-BIOTEK microplate reader.

**Cell lysate preparation**

For preparation of cell lysate, the treated and untreated cells collected, washed with cold PBS, pH 7.4 and were lysed in the ice-cold lysis buffer [50 mM Tris–HCl, pH 7.4, 0.5% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA] that contained protease inhibitors. Allowed to cells to stand on ice for 30 min and were vortexed every 10 min. The resulting mixture was centrifuged at 14,000 ×g for 15 min at 4 °C. Protein concentrations of supernatants were assayed using Bradford protein assay.

**Determination of malondialdehyde**

Malondialdehyde (MDA) is the end products of polyunsaturated fatty acids (PUFAs) peroxidation in the cells. This reactive aldehyde is one of important indicators of oxidative stress. MDA quantified by the thiobarbituric acid reactive substance (TBARS) method. Briefly, the cells were seeded in 3×10^5 per well in a 6-well plate, after overnight incubation, the cells were treated with different concentrations of trastuzumab for 48 h. After incubation time, cell lysis prepared and 500 μL of cell lysates were mixed with 1 mL of 10% (w/v) cold TCA to precipitate proteins. 1 ml 0.67 % (w/v) thiobarbituric acid (TBA) added to supernatant and heated at 95°C for 1h. The pink color product (MDA- TBA complex) absorbance was determined at 532 nm spectrophotometrically. Lysates protein concentrations determined by Bradford method and MDA concentration of the samples were calculated using following formula: Absorbance at 532nm / 1.56 x 105 -1cm^-1 expressed as nmol/mg protein.

**Determination of Protein carbonylation**

Protein carbonyls (PCOs) are common index of oxidative modification of proteins. Protein carbonyl contents quantified by the reaction between 2, 4-dinitrophenylhydrazine (DNPH) and protein carbonyls producing Schiff base to convert hydrazine derivatives determined spectrophotometrically at 360-385 nm. Briefly, the cells seeded in 3×10^5 per well in a 6-well plate, after overnight incubation, the cells were treated with different concentrations of trastuzumab for 48 h. After incubation time, the cells trypsinized and cell lysis prepared. 900 μL of cell lysates were mixed with 100 μL 10% (w/v) streptomycin solution. After 15 min standing and centrifugation (5000 rpm ×10 min), 10 mM DNPH in 2N HCl was added to supernatant. After 1hr at room temperature and vortexing every 10 min, 2ml TCA (10%w/v) was added and centrifuged (3000 rpm, 10min). Percipitate washed twice with 4ml ethanol/ethyl acetate (1:1, v/v), then 1ml guanidine hydrochloride (6M, pH = 2.3) added and vortexed. Results are reported as nmol with beer-Lambert formula (ε <sub>DNPH</sub> = 2.29×10^4 cm^-1M^-1).

**Statistical analysis**

Each experiment was carried out in triplicate and all experiments were performed three times. Results are expressed as means ± standard deviation (SD) and statistical analysis was performed using SPSS (version 16). Differences between two groups were tested using Student’s t test and P value < 0.05 were considered statistically significant.

**RESULTS**

**Effect of trastuzumab on cell viability**

The MTT assay was performed to determine the effect of trastuzumab on various breast cancer cells proliferation. Cells were treated with different doses of trastuzumab. As shown in Fig.1 there were significant cell proliferation inhibition ratio between each treated and untreated cells (p value <0.05). Trastuzumab treatment of various breast cancer cell lines cause dose dependent reduction in cell viability and proliferation inhibitory effect of trastuzumab was calculated. IC50 values of cells were analyzed using CompuSyn software version 0.1.

**Determination of intracellular reactive oxygen species (ROS)**

Several oxidative and enzymatic reactions in mitochondria, endoplasmic reticulum and peroxisome lead to generation of ROS. A variety of tests have been developed for ROS detection. In present study concentration of intracellular reactive oxygen species (ROS) was determined by...
DCFH-DA probe and fluorescence intensity was measured by 96 well plate spectrofluorometer. DCF fluorescence intensity correlated with ROS concentration of cells. Fig-2 shows the relative DCF-fluorescence intensity (mean±SD, N=3) in BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231 and MCF-7 breast cancer cell lines after 48 hours treatment with increasing concentrations of trastuzumab (up to IC50) and dose-dependent curve was drawn.

**Determination of lipid peroxidation**
Malondialdehyde as a major degradation product of lipid peroxidation was determined by the thiobarbituric acid (TBA) test. This spectrophotometrically method is based on the reaction of MDA and two molecules of TBA to form pink coloured complex (TBA$_2$-MDA) with a maximum absorption at 535 nm. The results are expressed as mean ± standard deviation of three independent experiments. (p<0.01). Fig-3 shows the Malondialdehyde levels (mean±SD, N=3) in BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231 and MCF-7 breast cancer cell lines after 48 hours treatment with increasing concentrations of trastuzumab (up to IC50) and dose-dependent curve was drawn.

**Determination of protein carbonyls (PCOs) content of cell proteins**
Reactive oxygen species also can oxidize proteins that results in inactivation of protein functions. Direct oxidation of lysine, arginine, proline and threonine (primary protein oxidation) or addition of reactive aldehyde to amino acid side chains (secondary protein oxidation), cause formation of reactive ketones that reacted by 2,4-dinitrophenylhydrazine (DNPH) to form detectable hydrazones. The results are expressed as mean ± standard deviation of three independent experiments. (p<0.01). Fig-4 shows the protein carbonyl levels (mean±SD, N=3) in BT-474, SK-BR-3, MDA-MB-453, MDA-MB-231 and MCF-7 breast cancer cell lines after 48 hours treatment with increasing concentrations of Trastuzumab (up to IC50) and dose-dependent curve was drawn.

<table>
<thead>
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<th>Cell lines</th>
<th>SK-BR-3</th>
<th>BT-474</th>
<th>MDA-MB-453</th>
<th>MDA-MB-231</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>2.02±0.36</td>
<td>1.54±0.21</td>
<td>5.34±0.69</td>
<td>3.63±0.71</td>
<td>5.20±0.54</td>
</tr>
</tbody>
</table>

**Figure 1: Measurement of cell proliferation and IC50**
A) for determination of cell proliferation, $10^4$ cell/plate seeded in 96-well plate. After an overnight incubation, cells washed twice with PBS and treated with various concentration of trastuzumab (0-10μg/ml) for 48 hours. Cells were then washed twice PBS and MTT test was carried out. B) The IC50 (50% of growth inhibition) of trastuzumab on various breast cancer cells were calculated by CompuSyn software. Data are representative of three independent experiments.
Figure 3: Effect of trastuzumab on Malondialdehyde production after 48 hours treatment: Malondialdehyde production in HER2 positive (SK-BR-3, BT-474 and MDA-MB-453) and HER2 negative (MDA-MB-231 and MCF-7) breast cancer cell lines induced by exposure to trastuzumab in dose dependent manner followed by thiobarbituric method. Values represent the mean value ± standard deviation of three independent experiments. *p<0.05 versus control.

Figure 4: Effect of trastuzumab on protein carbonylation after 48 hours treatment: Protein carbonyls levels in HER2 positive (SK-BR-3, BT-474 and MDA-MB-453) and HER2 negative (MDA-MB-231 and MCF-7) breast cancer cell lines induced by exposure to trastuzumab in dose dependent manner followed by DNP method. Values represent the mean value ± standard deviation of three independent experiments. *p<0.05 versus control.
DISCUSSION

Overexpression of the HER2/neu protein is observed in approximately 30% of breast cancer and has been associated with increased tumor cell proliferation, tumor invasiveness, progressive regional, distant metastasis and poor prognosis. Trastuzumab inhibits tyrosine kinase activity of HER2 receptor and usually offered for treatment of women with HER-2 positive breast cancer. Despite of initial response, women with HER-2 (ErbB-2) overexpressing metastatic breast cancer, not response trastuzumab treatment. The mechanisms of resistance to trastuzumab treatment are poorly understood, thus understanding this problem is essential to development of efficient chemosensitizer drugs [12, 13].

Cancer drug resistance is a complex phenomenon and major cause of treatment failure in cancer chemotherapy. Recent studies show that the mechanism of trastuzumab resistance is thought to be associated with cell cycle inhibition and co-expression of growth factor receptors [14].

Activation of HER2 receptor results in dimerization and activation of tyrosine kinase activity followed by phosphorylation of specific tyrosine residues that lead to the activation of downstream pathways, such as RAS-RAF-MEK-ERK1/2, PI3k-AKT-mTOR, SRC, PLC, PKC, STAT with various biological effects [15]. Hyperactivation of the PI3K/Akt pathway by mutations or loss of PTEN expression has been associated with resistance to trastuzumab-based chemotherapy and PTEN loss may be a marker of trastuzumab resistance. Other proposed markers of trastuzumab resistance include a truncated form of HER2 (p95), PIK3CA mutations, HER2/IGF-IR dimerization and Src activation [16]. Most anticancer agents induces apoptosis via activation of different genes that cause activation of master regulatory genes that lead to generation of Reactive Oxygen Species (ROS) in respective cancer cells[17]. Generation of ROS after treatment with chemopreventive agents affect several redox sensitive signaling pathways leading cell death [18]. Prolonged treatment with the same drug stimulates cellular protective antioxidant enzymes to cope with oxidative stress. These adaptation processes and decrease in cellular ROS levels involve activation of redox sensitive survival proteins via activation of antioxidant enzymes that lead to increase cell survival capacity that convert drug sensitive cancer cells into drug resistant cells [19].

Although there have not been any published studies on the oxidative effects of trastuzumab on SK-BR-3, BT-474 and MDA-MB-453 HER2 overexpressing breast cancer cell lines. In present study we examined SK-BR-3, BT-474 and MDA-MB-453 for trastuzumab-mediated oxidative stress. Our data revealed that trastuzumab as a monoclonal antibody binds to the extracellular domain of HER2 and change oxidative stress markers. According to our results, trastuzumab caused significant increase in reactive oxygen species in SK-BR-3, BT-474 and MDA-MB-453 cell lines (Fig2). Furthermore, increasing in malondialdehyde seen in HER2 overexpressing SK-BR-3, BT-474 and MDA-MB-cell lines after treatment with trastuzumab (Fig3). Fig4 shows that trastuzumab treatment caused significant increase in cellular protein carbonylation in SK-BR-3, BT-474 and MDA-MB-453 HER2 overexpressing breast cancer cell lines.

Dogan et al reported that inhibition of HER2 receptor by trastuzumab decreased intracellular glutathione levels, superoxide dismutase (SOD) and catalase (CAT) activities [20]. Treatment of isolated cardiomyocytes with trastuzumab induced cell death by increasing production of reactive oxygen species [21]. Pentassuglia and his coworkers showed that inhibition of HER2 by trastuzumab or with Paclitaxel increased oxidative stress in adult ventricular myocytes [22]. While administration of exogenous Neuregulin-1β (NRG) a ligand of cardiac HER2 receptors, reduced peroxide- or doxorubicin induced oxidative stress [23]. Mohan et al that trastuzumab but not Pertuzumzab, dysregulates HER2 signaling to mediate inhibition of outophagy and increase in reactive oxygen production in human cardiomyocytes [24]. Results show that inhibition of neurogulin-1 signaling by trastuzumab, blocks dimerization of receptor, resulting in NADPH oxidase activation and superoxide anion production but activation of HER2 dimerization by Neuregulin promotes mitochondrial changes by PI3/Akt signaling that lead to decreasing of ROS production [25].

CONCLUSION

Increasing of oxidative stress markers in HER2 overexpressing compared to HER2 negative breast cancer cell lines after treatment with trastuzumab
can be exploited for selective HER2 positive cancer cells therapy; combination of trastuzumab with ROS producing agents renders them more susceptible and induces cell death.

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Author details
1 Department of Clinical Biochemistry, Tarbiat Modares University, Tehran, Iran
2 Department of Hematology, Tarbiat Modares University, Tehran, Iran
3 National Institute of Genetic Engineering and Biotechnology

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