ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Trastuzumab Augments Apoptotic Cell Death of MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

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Abstract

Trastuzumab is a tyrosine kinase inhibitor used as a humanized monoclonal antibody to target HER2 amplified and/or overexpressed breast cancer cells. We aimed in the present research to evaluate the efficacy of trastuzumab to enhance apoptotic effect and DNA damage in normal and/or low-expressed HER2 of MCF-7 and MDA-MB-231 breast cancer cell lines. We evaluated the cytotoxic effect of trastuzumab on both cell lines after 6, 12, 24 and 48h. Then, we used the IC_{50} value related to each cell line after 24h to assess the apoptotic-necrotic effect, DNA damage using micronucleus test and screening immunocytochemistry of caspase-3 antibody as one of the apoptotic proteins.

Trastuzumab induced cytotoxic effect on both cell lines in a concentration dependent manner; however, this cytotoxicity was reduced by increasing time interval from 6 to 48h. The IC_{50} value reached to 17.56µg/ml and 26.15µg/ml for MCF-7 and MDA-MB-231 cells respectively after 24h. The mean percentages of cell death through apoptosis and induction of micronuclei were significantly increased ($p<0.001$) by raising the concentration of trastuzumab on both cell lines. Caspase-3 antibody was overexpressed in cancer cell treated with trastuzumab in a concentration dependent regarding that observed in non-treated cells.

High concentrations of trastuzumab raised cytotoxicity, caspase-3 protein expression and DNA damage of MCF-7 and MDA-MB-231 cells which consequently elevated apoptotic cell death remarkably. Further investigations are required to understand its mode of action to activate apoptotic effect on low-expressed breast cancer cells.

Keywords: Trastuzumab; Cytotoxicity; DNA damage; Micronucleus test; Apoptosis-necrosis. Caspase-3.

1. INTRODUCTION

The epidermal growth factor receptor family comprises many tyrosine kinases including ErbB1 (EGFR/HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) receptors, which play important role in cell growth through signal transduction and protein phosphorylation pathways [1-3]. Once any of tyrosine kinases become highly activated and independent of ligands due to molecular sequences mutations or protein over-expression, the cells become altered and proliferate extensively through unregulated processes leading to initiation of cancers [3-5]. Therefore, developing tyrosine kinase inhibitors (TKIs) was essential to target tumor cells, regulate cell growth processes and downregulate tumor progression in different cancer types either alone or in combination with other chemotherapies and/or radiotherapy [5].

Trastuzumab, humanized monoclonal antibody, is one of TKIs that bind to HER2 extracellular domain [6,7]. Food and drug administration (FDA) approved trastuzumab as a target therapy for breast cancer [8]; due to its potential to successfully suppress cancer cell growth, proliferation and survival through direct and indirect pathways [9-11]. Earlier clinical studies demonstrated that cancer patients who have trastuzumab in combination with other chemotherapies get better response rather than those who received trastuzumab alone [10,12], especially for breast cancer patients with HER2 over expression [13,14].

Earlier studies reported that the percentages for HER2 protein overexpression with or without gene amplification in breast cancer patients are ranging from 20-30% of all breast cancer cases [15-19]. Bose *et al.* [20] reported that the amplified HER2 genome wild sequences could include many mutations as alternate mechanism for HER2 activation in breast cancer. Previous research studies found that MCF-7 cell line has three HER2 gene copy numbers [21-23], which is not grade to amplification genotype as observed in other breast cancer cell lines such as SKBR3 [21,22,24]. MDA-MB-231 cell line has non-amplified HER2 genotype [21,22,24]. Both MCF-7 and MDA-MB-231 cell lines have moderate to low HER2 protein expression ($HER2^{+}$) respectively [25,26].

In the present basic research, we used MCF-7 and MDA-MB-231 cells to provide insight into the biological activity of trastuzumab to activate mode of cell death by investigating its cytotoxic effect, apoptotic-necrotic effect, induction of micronuclei as a monitor for DNA damage, and activation of caspase-3 as a marker for apoptotic pathway.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Trastuzumab (Herceptin) was purchased from Roche, Switzerland. Other buffers and reagents were obtained as analytical grades unless referenced.

2.2. Cell culture

Both human breast cancer cell lines "MCF-7 and MDA-MB-231" were delivered from the American Type Culture Collection (ATCC, VA, USA), routinely cultured in dulbecco's modified eagles medium (DMEM) containing 10% fetal bovine serum (Biowest) and antibiotic/antimycotic (Biowest) in humidified air chamber containing 5% CO2 at 37°C. Experiments were repeated three independent times (n=3). The mean percentages of cell viability were recorded as mean ± SE.

2.3. Cytotoxicity

We used 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) to assess cytotoxic effect of trastuzumab on breast cancer cell lines. Cytotoxicity was estimated using the 3-[4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT assay is depending on cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells [27]. MCF-7 and MDA-MB-231 cells were re-cultures in 96-well microplates (5x103 cell/well) and treated with different concentrations of trastuzumab for different time intervals (6, 12, 24 and 48h) to assess cytotoxicity. The mean percentage of treated viable cells were compared to non-treated cells to record the relative percentage of cell viability for each cell line and calculate its IC_{50} concentration. Then, IC_{50} value after 24h related for each cell line was used to conduct other bio-assays.

2.4. Apoptosis and Necrosis assay

Both cell lines were re-cultured on cell culture slides ($1x10^4$ cells/ well in 8 chamber slides-30108 SPL, South Korea) and treated with trastuzumab for 24h at concentrations 0.88, 1.75, 8.76 and 17.53µg/ml for MCF-7, and 1.31, 2.62, 13.08 and 26.16µg/ml for MDA-MB-231 cell lines. The mode of the cell death was investigated in non-treated and treated cells after staining with acridine orange (100µg/ml)/ ethidium bromide (100µg/ml) dye dissolved in phosphate buffer saline (PBS) [28]. The cell uptake of the stain was monitored under a fluorescence microscope (AxioImager Z2, Zeiss, Germany) with magnification power 20X. Cells with green fluorescence light were scored as live cells, and those with yellow or orange colors were recorded as apoptotic or necrotic cells respectively.

2.5. Micronucleus test

Both cell lines were re-cultured on the cell culture slides for 72h. The cultured cells were treated with cytochalasin B (C6762, Sigma, Schnelldorf) for 44h before the end of the experiments according to D'Souza *et al.* [29] with some modifications. Both cell lines were treated with trastuzumab for 24h at concentration 0.88, 1.75, 8.76 and 17.53µg/ml for MCF-7; and 1.31, 2.62, 13.08 and 26.16µg/ml for MDA-MB-231, then, the slides smears were fixed in absolute methanol for 10min and stained with 1µg/ml DAPI (D9542, Sigma, Schnelldorf). Scoring of thousand binucleated cells with or without micronuclei for each sample was recorded using a fluorescence microscope (AxioImager Z2, Zeiss, Germany) with magnification power 40X. Finally, the average percentages of micronuclei per experiment were recorded.

2.6. Immunocytochemistry of Caspase-3

We cultured MCF-7 and MDA-MB-231 cells on cell culture slides ($1x10^4$ cells/ well in 8 chamber slides- 30108 SPL, South Korea) with different concentrations of trastuzumab for 24h (0.88, 1.75, 8.76 and 17.53µg/ml for MCF-7, and 1.31, 2.62, 13.08 and 26.16µg/ml for MDA-MB-231). Slides were rinsed briefly with PBS, dehydrated and fixed with absolute methanol for 10min at room temperature, then blocked with 3% BSA in PBST (PBS-0.2% Tween) for 1h, followed by PBS washing. The slides were treated with 1/100 diluted anti-Caspase-3 primary antibody (Abcam, ab59388, Rabbit polyclonal ab) at 4°C overnight. The slides were washed thoroughly with PBS buffer and incubated at dark with goat pAb anti-rabbit IgG H&L (Phycoerythrin) as a secondary antibody (Abcam ab97070) in room temperature for 45min. The slides were washed with PBS, air drayed, counterstained with DAPI (1µg/ml), then examined under fluorescence microscope (AxioImager Z2, Zeiss, Germany) with magnification power 20X. Expression of caspase-3 was scored according to the HercepTest instructions [30]. Samples were categorized for caspase-3 expression according to the staining of the treated cells in comparison to non-treaded cells. If the majority scored cells of the sample were stained with complete and strong membrane staining in >10% of scored cells, it would be considered to be strong positive expression at grade 3^+. If stained cells were moderate to weak expressed in >10% of the cells, the sample would be considered positive at grade 2^+. If the scored cells were stained with incomplete membrane staining in >10% of the cells, then, the sample would be considered positive at grade 1^+. Finally, if there is no membranous staining in <10% of test cells, then, the sample would be considered to have no expression at all (grade 0).

Statistical analysis

GraphPad Prism software-V6 was used to assess the significance of data for cytotoxicity, apoptosis-necrosis and micronucleus test using One-way ANOVA-Dunnett's multiple comparisons test. Data were considered significant when $p<0.05$ for the mean percentages ± SE values.

3. RESULTS

3.1. Cytotoxicity

Our results indicated that trastuzumab induced cytotoxicity in MCF-7 and MDA-MB-231 cells at high concentrations. IC_{50} values differed according to the cell line type and varied according to the incubation time (Figure 1). For MCF-7 cells, the IC_{50} values ranged from 31.65, 21.08, 17.53 to 11.97 µg/ml regarding incubation times 6, 12, 24 and 48h respectively (Figure 1a).

For MDA-MB-231 cell line, the cytotoxicity values were slightly higher and IC_{50} values reached to 32.09, 29.07, 26.16 and 16.64µg/ml respectively to the same times-intervals (Figure 1b). Trastuzumab did not induce cytotoxic effect on MCF-7 or MDA-MB-231 cells at low concentrations (1.25 and 5µg/ml) along all-time intervals, however, it induced higher cytotoxicity at higher concentrations (10 and 20µg/ml). The IC_{50} values after 24h and lower related concentrations for each cell line were used in the followed bio-assays studies.

3.2. Apoptosis and Necrosis

We used the IC_{50} and lower equivalent concentrations for each cell line to assess the apoptotic-necrotic effect of trastuzumab after 24h on MCF-7 and MDA-MB-231 cells (Figure 2a,b). We discerned that the lower concentrations on each cell line (0.88µg/ml for MCF-7, 1.31 and 2.62µg/ml for MDA-MB-231) triggered apoptosis with no significance in comparison with related non-treated cells. The percentages of apoptotic cells were increased gradually in a significant manner by elevating trastuzumab concentration on both cell lines. Apoptotic cells were extremely significant ($p<0.001$) at concentrations 17.53 and 26.16µg/ml for MCF-7 and MDA-MB-231 respectively (Table 1), and their percentages reached to 38.67% and 30.67%.

The necrotic cells were increased regularly by raising trastuzumab concentrations, and percentages of necrotic cells reached to 19.33% and 17.67% ($p<0.01$) at the highest concentrations (17.53 and 26.16µg/ml for MCF-7 and MDA-MB-231) respectively. Figure 2c-h showed photomicrographs for MCF-7 and MDA-MB-231 cells treated with trastuzumab presenting live (green), apoptotic (yellow) and necrotic cells (orange).

3.3. Micronucleus

Both cell lines were treated with trastuzumab for 24h to assess DNA damage response using micronucleus test (Table 1). The mean percentages of binucleated cells containing micronuclei were increased frequently by increasing trastuzumab concentrations. In MCF-7 cells, the percentages of micronuclei were increased from 3.9% and 6.7% ($p>0.05$) at concentrations 0.88 and 1.75µg/ml to extreme significance 10.7% and 15.77% ($p<0.001$) at concentrations 8.76 and 17.53µg/ml in comparison with 4.27% for non-treated cells (Figure 3a).

In MDA-MB-231 cells, the mean percentages of micronuclei were raised gradually by elevating the concentration from 1.31, 2.62, 13.08 and 26.16µg/ml (Figure 3b). The mean percentages of binucleated cells were elevated non-significantly from 4.10%, 5.73% at lower concentrations to 8.17% ($p<0.01$) and 13.53% ($p<0.001$) at higher concentrations respectively regarding 3.43% for normal binucleated cells. Figure 3c-f presented photomicrographs for MCF-7 and MDA-MB-231 binucleated cells with/ without micronuclei.

3.4. Immunocytochemistry of Caspase-3

Both cell lines showed caspase-3 protein expression with low intensity (grade 1^+) before applying trastuzumab (Figure 4). When both cell lines treated with trastuzumab, caspase-3 remained with low expression (grade 1^+) at the lower concentrations (0.88 and 1.75µg/ml for MCF-7 and 1.31 and 2.62µg/ml for MDA-MB-231) and increased gradually to reached to grade 2^+ by elevating the concentrations to 8.76 and 13.08µg/ml for MCF-7 and MDA-MB-231 respectively at where more than 10% of treated cells contained partial membrane staining of caspase-3 protein. When both cell lines treated with higher concentrations of trastuzumab (17.53µg/ml for MCF-7, 26.16µg/ml for MDA-MB-231), the ratios of caspase-3 expression were strengthened extensively to complete cellular membrane in more than 10% of both continued survived cultured cell lines that caspase-3 expression reached to grade 3^+ (Table 1).

Table 1: Evaluation of apoptosis-necrosis, micronuclei in binucleated cells and caspase-3 protein expression after treating MCF-7 and MDA-MB-231 cell lines with trastuzumab for 24h.

Conc. (µg/ml)	Apoptosis-necrosis effect (Mean±SE)			MN in binucleated cells Mean(%)±SE	Caspase-3 expression Grades #
	Live cells	Apoptotic cells	Necrotic cells		
MCF-7					
0	89.33±3.76	4.33±1.76	6.33±2.03	4.27±0.41	+
0.88	82.67±4.16 ns	9.67±1.45 ns	7.67±2.85 ns	3.90±0.53 ns	+
1.75	70.33±2.73 **	18.00±2.89 **	11.67±0.88 ns	6.70±0.61 ns	+
8.76	53.00±2.08 ***	31.67±1.33 ***	15.33±2.73 *	10.70±0.57 ***	++
17.53	42.00±2.08 ***	38.67±2.19 ***	19.33±1.86 **	15.77±0.90 ***	+++
MDA-MB-231					
0	90.00±3.46	4.67±2.73	5.33±2.33	3.43±0.55	+
1.31	88.33±3.76 ns	6.00±1.73 ns	5.67±2.03 ns	4.10±0.61 ns	+
2.62	74.67±4.41 ns	15.00±3.21 ns	10.33±1.76 ns	5.73±0.52 ns	+
13.08	61.67±4.06 **	24.00±3.06 **	14.33±1.76 *	8.17±0.64 **	++
26.16	51.67±2.91 ***	30.67±3.18 ***	17.67±2.19 **	13.53±0.93 ***	+++

*p<0.05; **p<0.01; ***p<0.001 Significance in comparison with non-treated cells.

Grades represents (%) of protein expression in treated cells (details in methodology).

4. DISCUSSION

In the present investigation, we found that trastuzumab affected on two moderate (MCF-7) to low-HER2 (MDA-MB-231) expression breast cell lines [26,31]. We found that trastuzumab induced regular cytotoxicy in both cell lines after 6, 12, 24 and 48h, however, the IC_{50} values were reduced by increasing time of exposure. On the other hand, the IC_{50} values after 24h were reached to 17.53µg/ml and 26.16µg/ml for MCF-7 and MDA-MB-231 respectively. This observation, "high IC_{50} value", was consistent with the findings of Charafe-Jauffret *et al.* [32], who demonstrated that trastuzumab less than 10µg/ml did not induce cytotoxicity in both cell lines. Furthermore, over-expressed HER2 breast cancer cells respond to trastuzumab at low concentration effectively, and the IC_{50} value was less than 5µg/ml [33]. Also, Patra *et al.* [34] reported that the rate of cancer cell proliferation was decreased inversely to an increase in the trastuzumab dosage which indicated dose-response activity.

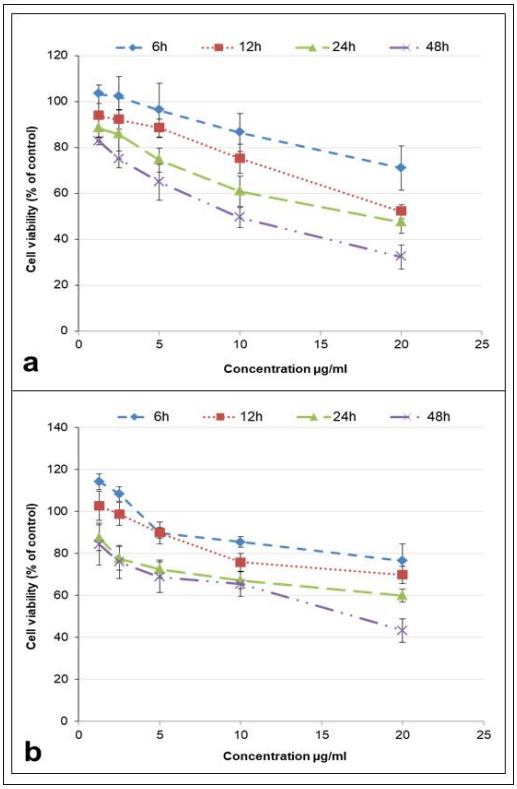

Fig. 1. Evaluation of trastuzumab cytotoxicity on (a) MCF-7 and (b) MDA-MB-231 breast cancer cell lines after treatment with variable concentrations for different time-intervals. Data are presented as mean (%) of control ± SE, n=3.

Although, trastuzumab efficacy is based on its interaction with HER2 receptors [6,7,26,35-37], we found that its effect reached to the cytogenetic levels and induced extensive genotoxicity. This was obvious when we found that trastuzumab generated cell death through apoptosis as well as micronuclei in binucleated cells of MCF-7 and MDA-MB-231 cells. This observed DNA damage was directly correlated with trastuzumab concentration. Previous reports demonstrated that trastuzumab acted as antitumor monoclonal antibody in breast cancer cells by upregulating cycline-dependent kinase inhibitor (p27Kip1), which consequently augmented cell cycle arrest [9,38,39]. Moreover, in a model of cellular-dependent cytotoxicity of glioblastoma cell lines [40] and in primary breast tumors [41] trastuzumab enhanced its cytotoxic and genotoxic effects through apoptotic pathway rather than cell cycle arrest. Some research studies attributed the pro-apoptotic and anti-proliferative activities of trstuzumab to the downregulation of transforming signals of HER2 including that of Raf/MEK/ mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3`-kinase (PI3K/Akt kinase pathways [42,43]. Moreover, PI3K/Akt kinase protein has a critical role in regulating many proteins including that involved in cell cycle, apoptosis, glycogen metabolism, protein synthesis [44], and p27Kip1 [38].

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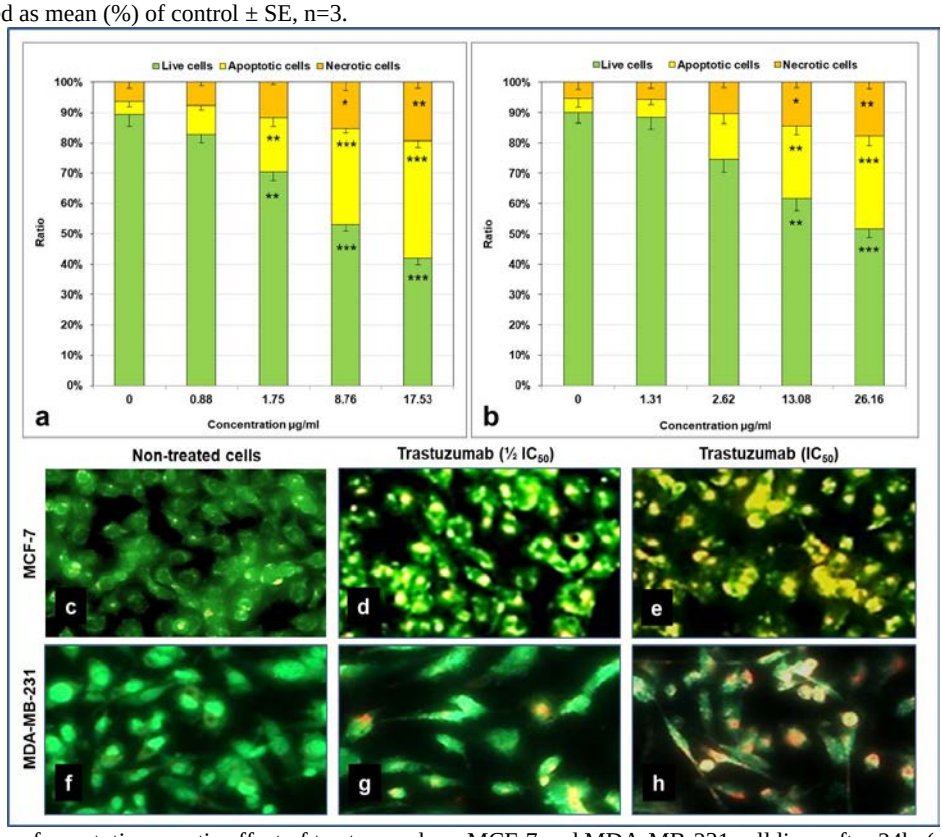

Fig. 2. Evaluation of apoptotic-necrotic effect of trastuzumab on MCF-7 and MDA-MB-231 cell lines after 24h. (a,b) illustration of mean percentages of apoptotic-necrotic effects on both cell lines respectively after different treatments; data represent means ± SE, n=3. (c,f) Photomicrographs for non-treated MCF-7 and MDA-MB-231 cells; (d,e) represent the effect of trastuzumab on MCF-7 at concentrations (½ IC_{50}: 8.76µg/ml and IC_{50}: 17.53µg/ml); (g,h) represent the effect of trastuzumab on MDA-MB-231 cells at concentrations (½ IC_{50}: 13.08µg/ml and IC_{50}: 26.16µg/ml); cells were stained with acridine orange/ethidium bromide dyes (v/v) and cells dyes illustrate live cells (green), apoptotic cells (yellow) and necrotic cells (orange) (x20).

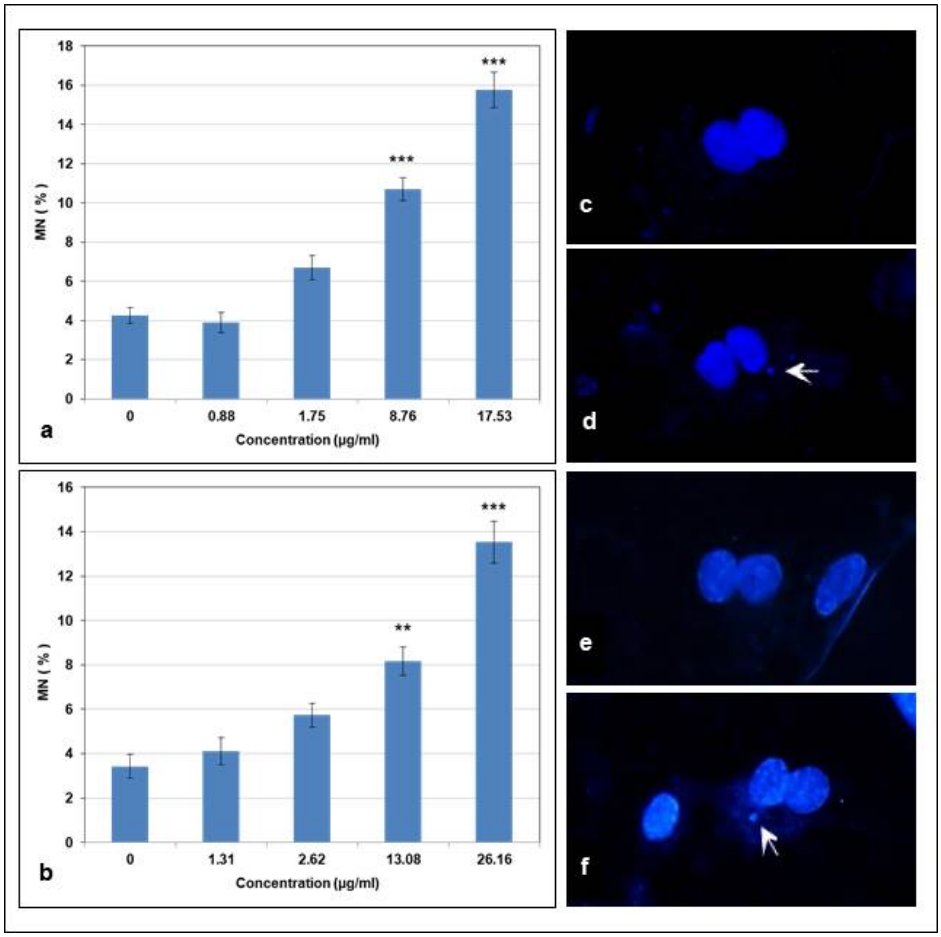

Fig. 3. Trastuzumab induced DNA damage as represented by micronucleus test on MCF-7 and MDA-MB-231 cells after 24h; (a,b) illustration of the mean percentages of micronuclei induced in binucleated cells of MCF-7 and MDA-MB-231 cells respectively; data represents means ± SE, n=3; (c,e) binucleated cells without micronuclei; (d,f) binucleated cells with micronuclei for MCF-7 and MDA-MB-231 cells respectivlely; cells were stained with DAPI (blue) (x20).

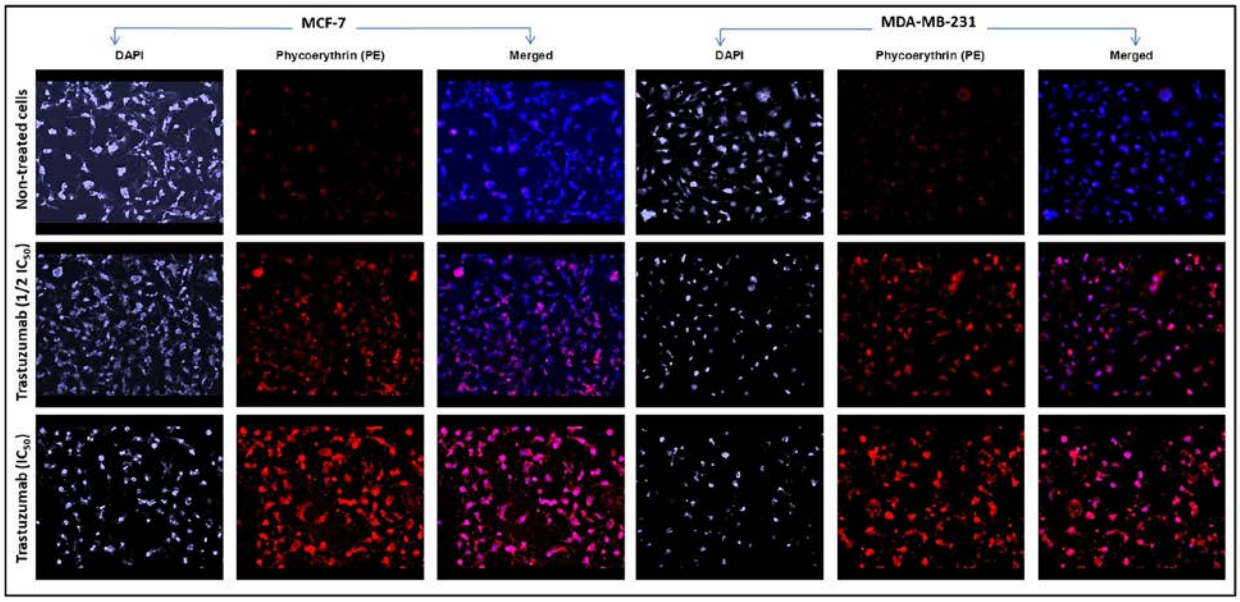

Fig. 4. Photomicrographs panel represents the effect of trastuzumab on MCF-7 (½ IC_{50}: 13.08µg/ml; IC_{50}: 26.16µg/ml) and MDA-MB-231 (½ IC_{50}: 8.76µg/ml; IC_{50}: 17.53µg/ml) to increase caspase-3 protein expression after 24h using immunecytofluorescence caspase-3 primary rabbit polyclonal antibody (ab59388) conjugated with goat pAb anti-rabbit IgG H&L (ab97070) labeled with phycoerythrin (red); the nuclei are stained with DAPI (images illustrate inverted DAPI or blue DAPI for merged slides) (x20).

We validated trastuzumab induction of apoptotic effect and enhancing DNA damage by investigating caspase-3 protein. It is well-known that caspases play a crucial role throughout apoptotic pathways [45,46]. From these caspases, caspase-3, which is responsible for most of apoptotic processes and could be activated by upregulating initiator caspases through extrinsic pathways by caspase-8 or intrinsic pathways by activating mitochondrial-dependent-cytochrome C/ particularly caspase-9 pathway [47-49]

We found that trastuzumab increased caspase-3 expression extensively by elevating its concentration on both MCF-7 and MDA-MB-231 cells. Therefore, we think that trastuzumab could activate apoptotic pathway, even in low or moderate HER2 expression breast cancer cells. Nagata *et al.* [50] reported that activating phosphatase and tensin homolog (PTEN) tumor suppressor protein could support the effectiveness of trastuzumab in breast cancer patients that achieved when trastuzumab binds to HER2 receptors to block PI3K signaling pathway leading to Akt dephosphorylation, PTEN tyrosine phosphorylation reduction, PTEN membrane localization accumulation, phosphatase activation and consequently inhibition of cell proliferation.

Earlier research studies reported that mechanistically trastuzumab does not block only signal transduction via HER2 surface receptor and its interaction patterns, but, it could enhance cell death in *in vivo* by stimulating immune-mediated response through activating cell mediated antibody-dependent cellular cytotoxicity (ADCC) [42,51,52], which activates natural killer (NK) cells and complement dependent cytotoxicity [53]. Then, NK cells could bind to trastuzumab via Fc gamma-receptor and activate lysis of cancer cells bound to trastuzumab [13,54,55]. Raab *et al.* [31] established the capacity of trastuzumab to induce ADCC in breast cancer cell lines. Also, the Ab-dependent cell-mediated cytotoxicity mediated by NK cells is well recognized as one of the key mechanisms of action for trastuzumab that macrophages by way of phagocytic engulfment, can mediate antibody-dependent cellular phagocytosis (ADCP) and cancer cell killing in the presence of trastuzumab [56].

5. CONCLUSIONS

Trastuzumab at high concentrations enhanced cell death of MDA-B-231 and MCF-7 cell lines. There was indirect correlation between IC_{50} concentration and time of exposure. When both cell lines were treated with trastuzumab, it activated apoptotic stress as evaluated by apoptosis-necrosis assay. Indeed, it increased micronuclei in scored binucleated cells; as well as Caspase-3 protein expression was directly increased by elevating trastuzumab concentration. Further investigations are required to understand the mechanistic role of trastuzumab to gradually enhance cytotoxicity and genotoxicity of MDA-MB-231 and MCF-7 as two low to moderate HER2-expression breast cancer cell lines.

Acknowledgements

This work was funded with grant no. 11010187 from the National Research Centre (NRC), Cairo, Egypt.

Conflict of Interest

Authors declared no competing of interest.

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