Synergistic Interaction of Lapatinib plus Doxorubicin or 5-Fluorouracil Accelerates Cell Death of Triple Negative Breast Cancer MDA-MB-231 Cell Line

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Abstract: Background: Lapatinib, one of tyrosine kinase inhibitor (TKIs), is used to treat HER1/2 amplified breast cancer diseases. Its combination with other chemotherapeutic agents was effective to diminish over-expressed-HER2 cell growth. This research aimed to assess its synergistic growth inhibition in combination with doxorubicin (DOX) or 5-fluorouracil (5-FU) on MDA-MB-231 as a model of human triple negative breast cancer (TNBC) cell line. **Methods:** 2.5%, 5% and 10% of lapatinib IC_{50} concentrations were tested in pre- and post-combinations with 10% of IC_{50} for DOX or 5-FU. Cytotoxic and genotoxic effects were conducted using MTT assay, apoptosis-necrosis and micronucleus (MN) tests. **Results:** Pre-treating MDA-MB-231 cells with DOX or 5-FU for 4h followed by lapatinib for 24h enhanced cytotoxicity ($p<0.05$) in comparison with that pre-treated with lapatinib for the same time intervals. Both pre- and post-treated MDA-MB-231 cells with lapatinib enhanced induction of apoptosis and DNA damage. The mean percentages of apoptotic cells and binucleated cells containing micronuclei were elevated remarkably ($p<0.05$) in cells pre-treated with DOX or 5-FU rather than that pre-treated with lapatinib. **Conclusion:** Synergistic interaction of lapatinib in combination with DOX or 5-FU augmented cell growth inhibition, apoptotic mode of cell death and DNA damage effectively. Additionally, the synergistic effect between chemotherapeutics and TKIs possibly will allow using lower concentrations to achieve remarkable cell death.

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1. Introduction

Triple-negative breast cancers (TNBCs) are varieties of tumors that lack expression of estrogen (ER), progesterone (PR) receptors and human epidermal growth factor receptor-2 (tyrosine kinase HER2 receptor) [1,2]. TNBCs are aggressive cancer subtypes that lack prognosis [3] and gain resistance to different chemotherapeutic drugs such as paclitaxel, vinorelbine, gemcitabine, doxorubicin or 5-fluorouacil after a series of treatments [4,5]. One of TNBCs is MDA-MB-231 cell line, which is mainly correlated to poor outcomes, showing the worst overall and disease-free survival rates due to lack of effective targeted therapies [6,7].

Doxorubicin (DOX), one of the most effective anthracycline components, is used in breast cancer regimens [8]. The activity of DOX returns back to its intercalation with the nitric bases of DNA double helix, generating free radicals that rupture DNA strands, inhibiting the respiratory chain enzymes in mitochondria, causing membrane lipid oxidation, interference with DNA unwinding and helicase activity and induction of apoptosis in response to topoisomerase II inhibition [9].

5-Fluorouracil (5-FU) is fluoropyrimidines, which is used as the first-line in different cancer regimens including breast cancer [10]. 5-FU is similar to pyrimidines incorporated into DNA and RNA, thus, it interferes with nucleoside metabolism causing cytotoxicity [11,12]. Also, it affects cell cycle regulation and causes cell cycle arrest through G1/S phase [13,14]. It generates mitochondrial reactive oxygen species (ROS), which might activate caspase-6 and p53-dependent apoptotic pathways [15,16].

Lapatinib (Lap), a reversible dual of tyrosine kinase inhibitors (TKIs), is homologous to the adenosine triphosphate (ATP). This inhibitor targets and inhibits selectively two receptors of human epidermal growth factor family (EGFR/ErbB1 and HER2/ErbB2) by preventing phosphorylation, activating signal transduction pathways, triggering apoptosis and decreasing cellular proliferation [17]. Lapatinib has been approved for metastatic HER2-positive breast cancer treatments. It was used in combination with letrozole, an aromatase inhibitor, as a first-line treatment to metastatic breast cancers with HER2 and hormonal over-expression [18]. Also, it was used in combination with different chemotherapies as a second-line for breast cancer patients primarily treated with different chemotherapeutics [19-21].

Therefore, it was important to seek for the suitable strategies enhancing cytotoxicity of TKIs and chemotherapeutics on MDA-MB-231 cells as one of the TNBCs cells. Hence, we assessed the synergistic therapeutic effects of lapatinib in combination with DOX or 5-FU on MDA-MB-231 cell line using cytotoxic assay, apoptosis-necrosis and micronucleus tests as DNA damage cytogenetic monitors.

2. Materials and Methods

2.1. Chemicals and reagents

Lapatinib (CDS022971; Sigma Aldrich, USA) and 5-Fluorouracil (F6627, Sigma, Schnelldorf) were solubilized in DMSO then diluted in deionized distilled water to final DMSO save concentration ≤0.1%. Doxorubicin (D1515, Sigma, Schnelldorf) was dissolved in deionized distilled water. Other buffers and reagents were obtained as analytical grades; unless mentioned.

2.2. Cell culture

The human breast cancer cell line MDA-MB-231 (American type culture collection; ATCC, VA, USA) was routinely cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (Biowest) and antibiotic/antimycotic (Biowest) at 37°C in humidified air chamber containing 5% CO_2. Monolayer cells were harvested by trypsin/ EDTA (Biowest) and re-cultured in microplates to assess cytotoxic and genotoxic assays.

2.3. Design of combined treatments

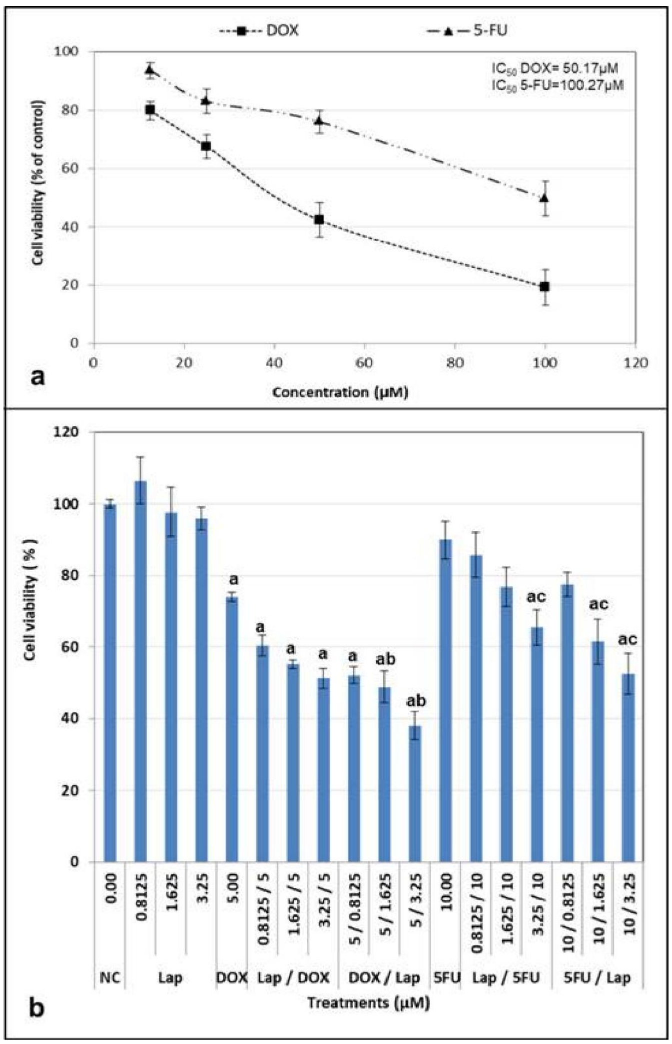

Figure 1: (a) Cytotoxicity of Doxorubicin (DOX) or 5-fluorouracil (5-FU) on MDA-MB-231 cells at variable concentrations with IC_{50}. (b) The combined treatments of 10% of IC_{50} of lapatinib (Lap), DOX and/or 5-FU on MDA-MB-231 cells after 24h using MTT assay. [a] $p<0.05$ significance in comparison to non-treated cells (NC); [b] $p<0.05$ significance in comparison to DOX; [c] $p<0.05$ significance in comparison to 5-FU. Data presented mean (%) ± SE; n=3.

Samples were collected 24h after the last treatments. Experiments were repeated three times independently and values of mean (%) ± SE were recorded.

The cytotoxic effects of DOX and 5-FU separately were evaluated on MDA-MB-231 cells after 24h to assess their IC_{50} concentrations. Together with our previous recorded lapatinib IC_{50} concentration on MDA-MB-231 cells for 24h (32.5µM- data not shown), cells were treated with combined chemotherapeutics at 10% of IC_{50} to conduct the cytotoxicity and genotoxicity as follows:

1st experiment: cells were treated with vehicle as negative control.

2nd, 3rd and 4th experiments: cells were treated with lapatinib, DOX or 5-FU respectively.

5th and 6th experiments: MDA-MB-231 cells were treated with lapatinib for 4h, then, they were treated with DOX or 5-FU separately.

7th and 8th experiments: cells were treated with DOX or 5-FU for 4h, then, they were treated with lapatinib.

2.4. Cytotoxicity

The MDA-MB-231 cells were incubated in 96 well plates ($5x10^4$ cells/ well) at variable concentrations of lapatinib, DOX or 5-FU, separately, or in combination before submitting them to 3-[4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Cytotoxic effect was estimated using MTT assay, which depends on cleavage of tetrazolium salt in the presence of mitochondrial dehydrogenase enzymes in vital cells [22]. The relative cell viability was expressed as the mean percentages of viable cells compared with non- treated cells.

Table 1: Mean percentages of live, apoptotic or necrotic MDA-MB-231 cells plus binucleated cells containing micronuclei (MN) after treatment with lapatinib (Lap), doxorubicin (DOX) and/or 5-fluorouracil (5-FU) for 24h.

Treatments	µM	Live cells	Apoptosis	Necrosis	MN
NC	0	89.67±2.68	4.18±0.88	6.15±0.67	0.53±0.09
Lap	3.25	83.67±3.49	9.39±1.45	6.95±0.88	1.50±0.12
Dox	5	72.67±2.03 [a]	18.71±2.03	8.63±0.91	2.23±0.22 [a]
Lap / Dox *	3.25 / 5	51.33±2.61 [ab]	35.33±5.16 [ab]	13.33±2.04	3.37±0.24 [ab]
Dox / Lap **	5 / 3.25	31.33±3.19 [ab]	38.98±4.16 [ab]	29.68±2.19 [ab]	4.37±0.26 [ab]
5-FU	10	77.33±3.01	13.36±1.20	9.30±1.46	1.77±0.32 [a]
Lap / 5-FU *	3.25 / 10	59.33±3.76 [ac]	24.93±4.41 [a]	15.73±0.88 [a]	2.57±0.18 [a]
5-FU / Lap **	10 / 3.25	46.67±2.49 [ac]	36.08±4.35 [ac]	17.25±2.48 [ac]	4.03±0.24 [ac]

[a] $p<0.05$ significance in comparison to non-treated cells (NC); [b] $p<0.05$ significance in comparison to DOX; [c] $p<0.05$ significance in comparison to 5-FU; * Cells were pre-treated with lapatinib for 4h followed by DOX or 5-FU. ** Cells were pre-treated with DOX or 5-FU for 4h followed by lapatinib. Data presented mean (%) ± SE; n=3.

2.5. Apoptosis-necrosis assay

The mode of the cell death was investigated in non-treated and treated cells after culturing them on cell culture slides (8 chambers/ slide- 30108 SPL, South Korea) and staining them with acridine orange/ ethidium bromide (AO/EB- 100 µg/ml; V/V) dissolved in phosphate buffer saline (PBS) [23]. The cell uptake of the stain was monitored under a fluorescence microscope (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.

2.6. Micronucleus (MN) test

MDA-MB-231 cells were cultured on 8-well spread chamber cell culture slides (30108 SPL, South Korea) and treated with cytochalasin B (C6762, Sigma, Schnelldorf) for 44h before the end of the experiments according to D'Souza et al. [24] with some modifications. MDA-MB-231 cells were treated with lapatinib, DOX and/or 5-FU according to the experimental design, then, they were fixed in absolute methanol for 10min and stained with 1µg/ml DAPI (D9542, Sigma, Schnelldorf). Scoring of a thousand binucleated cells with or without micronuclei for each sample was recorded using a fluorescence microscope (Zeiss, Germany) with magnification power 40X. Finally, the average percentages of micronuclei per experiment were recorded.

Statistical analysis

GraphPad Prism software-V6 was used to assess the significance of different bioassays by one-way ANOVA-Tukey's multiple comparisons test. Data were considered significant when $p<0.05$.

3. Results

3.1. Cytotoxicity

The TNBC cells "MDA-MB-231" were treated with variable concentrations of DOX and 5-FU for 24h and calculated their IC_{50} values through linear extrapolation. The IC_{50} concentrations of DOX and 5-FU were 50.17µM and 100.27µM respectively (Figure 1a).

When MDA-MB-231 cells were treated with 2.5%, 5% and 10% of lapatinib IC_{50} (equivalent to 0.8125, 1.625 and 3.25µM respectively), the mean percentages of viable cells were reduced non-significantly in comparison to non-treated cells. This applied also to the 10% of 5-FU IC_{50} (10µM), but the 10% of DOX (5µM) reduced cell viability significantly at $p<0.05$ (Figure 1b).

In the combination treatments, when breast cancer cells were treated with lapatinib for 4h then treated with DOX or 5-FU, the mean percentages of cell viability were reduced by increasing the concentration of lapatinib regarding non-treated cells. The remarkable reduction of cell viability was recorded when MDA-MB-231 cells were treated with DOX or 5-FU for 4h, then treated with 5% and 10% of lapatinib IC_{50} for extra 24h that cytotoxicity was elevated highly significant ($p < 0.05$) in comparison to non-treated cells, DOX or 5-FU separately (Figure 1b).

3.2. Apoptosis-necrosis assay

Establishing mode of cell death induced by synergistic interaction of lapatinib plus DOX or 5-FU at 10% of IC_{50} concentrations was illustrated using apoptosis-necrosis assay (Table 1). The combined treatments elevated cell death extremely significant regarding non-treated cells or that treated with lapatinib (3.25µM), DOX (5µM) or 5-FU (10µM).

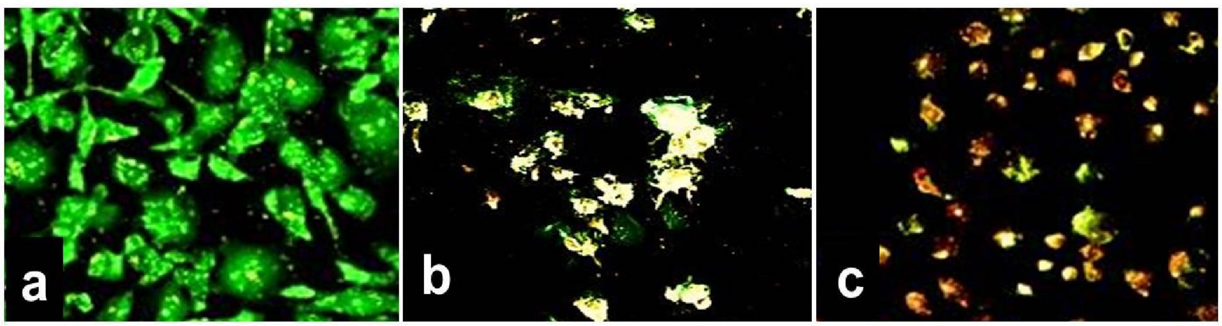

Figure 2: Photomicrographs for MD-MB-231 cells after combined treatments of lapatinib, doxorubicin and/or 5-fluorouracil illustrating mode of cell death through (a) live, (b) apoptotic and (c) necrotic cells.

Pre-treating cells with lapatinib for 4h followed by DOX or 5-FU enhanced cell death significantly through apoptosis (35.33% and 24.93%; $p<0.05$) rather than that died through necrosis (13.33% and 15.73%) respectively. The remarkable cell death was recorded when MDA-MB-231 cells were treated with DOX or 5-FU for 4h then treated with lapatinib that the apoptotic cells reached to 38.98% and 36.08% and that of necrosis were 29.68% and 17.25% respectively. Evaluating treated cells stained with AO/EB revealed that vital cells (green), apoptotic cells (yellow) and necrotic cells (orange) as investigated under fluorescent microscope (Figure 2a-c).

3.3. Micronucleus (MN) test

The mean percentages of DNA damage using micronucleus test were investigated after treating cancer cells with single or combined therapeutics of lapatinib DOX or 5-FU. Figure (3) represents examples of scored binucleated MDA-MB-231 cells with or without micronuclei.

When MDA-MB-231 cells were treated with lapatinib (3.25µM), the mean percentage of binucleated cells with micronuclei was increased non-significantly to 1.5% in comparison to 0.53% for non-treated cells.

In combined treatment, pre-treating MDA-MB-231 cells with lapatinib for 4h prior to DOX (5µM) or 5-FU (10µM) increased the mean percentages of binucleated cells with micronuclei to 3.37% or 2.57% respectively. Moreover, the highest DNA damage percentages were observed when cells were treated with DOX or 5-FU for 4h prior to lapatinib. The mean percentages of binucleated cells with micronuclei were reached to 4.37% or 4.03% in comparison to DOX (2.23%) or 5-FU (1.77%) respectively (Table 1). Therefore, combined treatments increased the mean percentages of DNA damage highly significant ($p<0.05$) in comparison with that of single treatments.

4. Discussion

Lapatinib is a dual alterable TKI for EGFR and HER2 receptors [17,25,26]. In MDA-MB-231 cell line, lapatinib performance reduced to half its power due to the low-expression of HER2 [2,27,28]. Therefore, this research study aimed to disrupt MDA-MB-231 cells with the combined synergistic interaction of lapatinib with DOX or 5-FU as two different types of chemotherapeutic agents. When MDA-MB-231 cells were treated with lapatinib for 4h prior to DOX or 5-FU, cytotoxicity was elevated significantly at 3.25µM of lapatinib regarding cells treated with DOX or 5-FU alone. However, the significant cell growth inhibition was demonstrated when MDA-MB-231 cells were pre-treated with the chemotherapeutics DOX or 5-FU. Many studies approved the efficacy of combined therapy of lapatinib with other chemotherapies or TKIs such as letrozole or trastuzumab resistant patients with over-expressed HER2 [18,21,29].

The mode of cell death with combined therapeutics was observed when MDA-MB-231 cells were pre-treated with DOX or 5-FU prior to lapatinib that apoptotic ratios were elevated remarkably to 9.3 and 8.6 folds respectively from control, in comparison to that of single treatments of lapatinib, DOX or 5-FU (2.2, 4.5 and 3.2 folds) respectively. But, when MDA-MB-231 cells were pre-treated with lapatinib followed by DOX or 5-FU, apoptotic cells were increased by 8 and 6 folds respectively. On the other hand, the proportion of necrotic cells in combined treatments reached to 4.6 and 2.8 folds for cells pre-treated with DOX or 5-FU prior to lapatinib comparing to those pre-treated with lapatinib (2.2 and 2.6 folds respectively). This elevated proportion of apoptosis might be returned back to the synergistic interaction of chemotherapeutics together with lapatinib that DOX could enhance apoptosis by activating p53- mediated upregulation of Noxa and Puma pathways that mediate caspase-9 and in consequence stimulate intrinsic apoptotic pathway [30]. Likewise, it might activate p53 and/or TNF signaling that activate caspase-3 and accordingly activate apoptotic pathways [31] and enhance DNA damage [32]. Furthermore, the potential of DOX to intercalate through DNA helix and release ROS [9] might activate its potential to induce apoptotic cell death remarkably. Similarly, interfering of 5-FU with pyrimidines could activate its incorporation inside DNA or RNA and consequently activate caspase 6 which might trigger apoptotic cell death [16] and arrest cell cycle at G1/S phase effectively [13,14].

On the other hand, the efficacy of lapatinib originates from its potential to bind to adenosine triphosphate (ATP) of epidermal growth family domains and prevent phosphorylation by blocking activated receptors [25], which downregulates protein kinase MAPK and PI3K/Akt pathways [33-35], and actively enhances BIM activation and survivin down regulation [36], which consequently inhibits cell proliferation and diminishes tumor growth. Furthermore, the synergistic interaction was observed in combined study of DOX together with TKIs with normal or overexpressed-HER2 (MCF-7 and MDA-MB-468 cell lines) that pretreating these cells with erlotinib or lapatinib prior to DOX activated caspase-8 and autocatalytic cleavage, which stimulated induction of cell death through extrinsic apoptotic pathway via death receptors [37].

This was obvious when induction of apoptosis led to orientation of DNA damage as recorded by micronucleus test. The percentages of pre-treated MDA-MB-231 cells with lapatinib former to DOX or 5-FU raised MN (%) by 6.3 and 4.8 folds respectively. The promising DNA damage ratios were achieved when cancer cells were pre-treated with DOX or 5-FU prior to lapatinib that MN (%) elevated with 8.2 and 7.6 folds respectively. Therefore, we could assume that treating TNBC cell lines with the suitable chemotherapeutic regimens could activate cell suicide and trigger apoptosis. Recently, many research studies were done on MDA-MB-231 cells as a TNBC subtype *in vitro* at low concentrations to assess the potential of combined chemotherapeutics to reduce proliferation, inhibit resistance, invasion and migration. The combinational treatments of furanodiene and DOX chemotherapies reduced invasion and migration *in vitro* [38]. Also, combined treatments of proanthocyanidins, isolated from *Uncaria rhynchophylla*, together with 5-FU activated cytotoxicity by releasing ROS and accelerating MDA-MB-231 apoptotic cell death [39]. The synergistic interaction between sulforaphane and 5-FU augmented MDA-MB-231 autophagy cell death after inhibiting cell growth effectively [40]. Besides, the efficiency of combined regimens between TKIs members was observed when lapatinib and foretinib affected EGFR and hepatocyte growth factor receptors (MET), respectively by reducing cell growth at G2/M phase, declining pAKT and inhibiting migration of TNBC cell lines [41].

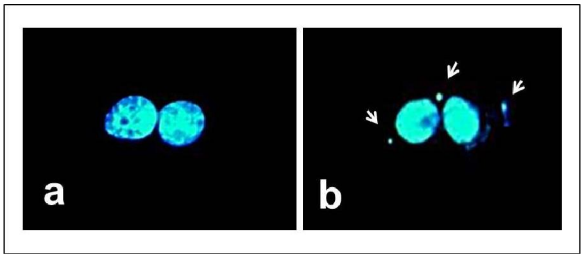

Figure 3: Photomicrographs for binucleated MDA-MB-231 cells after treating them with lapatinib, doxorubicin and/or 5-fluorouracil and recording (a) normal binucleated cells, and (b) binucleated cells with micronuclei.

5. Conclusion

The combinational regimens between DOX or 5-FU chemotherapeutics followed by TKIs, particularly lapatinib, augmented cytotoxicity of MDA-MB-231 cells and accelerated apoptotic cell death and DNA damage at low concentrations efficiently. Further investigations are required to reach to the optimum synergistic schedules between tyrosine kinase inhibitors and chemotherapeutic that could trigger triple-negative breast cancer cell death.

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