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The use of a combination of tamoxifen and doxorubicin synergistically to induce cell cycle arrest in BT483 cells by down-regulating CDK1, CDK2 and cyclin D expression

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Abstract

Background: Tamoxifen and Doxorubicin are used alone or in combination to treat breast cancer. Although these drugs have been utilized in combination, the advantage of their combination, in terms of therapeutic efficacy, still remains controversial.

Methods: Cells were treated with Tamoxifen alone and doxorubicin alone or treated with both Tamoxifen and doxorubicin, cell cycle distribution, cell cycle regulatory protein, mRNA, and activity were measured.

Results: This study uses breast cancer cell lines to demonstrate the synergistic interaction between Doxorubicin and Tamoxifen, and to explain the CDK1 and CDK2 expression underlying the synergy. This study demonstrates that the combination of Doxorubicin and Tamoxifen significantly reduces the growth of ER-positive breast cancer cells and that this is driven primarily by the enhanced effect of the decreased protein expression of the CDK1, CDK2 and cyclin D. It is also proposed that selective modification of AKT inactivation, ERK activation probably contributes to the synergistic interaction.

Conclusions: Overall, the findings suggest that a combination of Doxorubicin and Tamoxifen could be effective in the treatment of ER-positive breast cancers, so this combination warrants further investigation.

Keywords: Tamoxifen, doxorubicin, BT483 cells, CDK1, CDK2

Background

Doxorubicin (Dox) and Tamoxifen (Tam) are both very effective in the treatment of breast cancer. Dox is one of the most effective agents available for metastatic breast cancer. It is classified as an anticancer anthracycline antibiotic and is commonly used in the treatment of multiple cancers, including bladder cancer, breast cancer, ovarian cancer, lung cancer and gastric cancer [1-4]. However, several randomized clinical studies have included this drug in adjuvant regimens, possibly because of a fear of long-term cardiotoxicity [5]. Tam is a nonsteroidal selective estrogen receptor modulator that is widely used in the treatment of hormone-dependent breast cancers. In the clinical environment, Tam is the most commonly used endocrine therapy for women with breast cancers that express estrogen receptors and has enhanced patient survival [6]. However, five-year Tam use has become associated with a number of serious side effects. Therefore, alternative interventions are needed to replace or to supplement current regimens. There are grounds for the belief that multiple drugs may be more effective in the treatment of breast cancer than a single drug. In accordance with this concept, the ideal regimen would contain at least two potent drugs [7]. For example, preclinical studies provide evidence that the combination of gefitinib and Tam results in a strong synergistic effect [8,9].

Deregulation of cell cycle regulation is a general ha-

llmark of tumor formation and progression. Cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors play a well-established role in cell cycle control and have been attracting considerable research attention in the past decade. CDK1 and CDK2 are both thought to play an important role in cell proliferation and are probably associated with tumor aggressiveness and a poor prognosis. Thus, the direct targeting of CDK1 and CDK2 may be a useful therapeutic approach for breast cancer patients. Recent studies have shown that combined CDK2 and CDK1 depletion induces cell cycle arrest in breast cancer cells [10,11]. This study seeks to investigate whether similar effects could be achieved by reducing CDK activity, through the combination of Dox and Tam treatment.

Tam is the drug of choice for the treatment of estrogen-receptor positive breast cancer. While Dox may be used after relapse in patients, after Tam treatment, these drugs have been used in combination, but the advantage of this combination, in terms of therapeutic efficacy, still remains questionable. This study uses breast cancer cell lines to demonstrate the synergistic interaction between Dox and Tam, and to explain the CDK1 and CDK2 expression underlying the synergy.

Materials and Methods

Cell culture

The human breast cancer cell lines used in this study were MDA-MB-231, and BT483. MDA-MB-231 and BT483

were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin, and maintained at 37°C in 5% CO₂ humidified air. The 231.neo and 231.eB transfectants, which were kindly provided by Dr. Mien-Chie Hung (The University of Texas, M.D. Anderson Cancer Center, Houston, TX), were cultured as previously described [12].

MTT assay

The effect of Tam, Dox, or NU6102 on cell growth was examined by MTT assay. Briefly, cells were seeded at 2×10^4 cells/well in a 24-well flat-bottomed plate for 24 h, treated with varying concentrations of Tam, Dox, or NU6102 and incubated for an additional 48 h. After incubation, 20 µl of MTT solution (5 mg/ml, Sigma Chemical Co.) was added to each well and incubated for 1 h at 37°C. The supernatant was aspirated and the MTT-formazan crystals formed were dissolved in 200 µl of dimethyl sulfoxide (DMSO), and measure the absorbance at 550nm.

Flow cytometry

For the flow cytometric analysis, cells were seeded on 10 mm petri-dishes for various times. After incubation, the cells were trypsinized, washed with PBS, and fixed in 100% ethanol. Then, 1 ml of propidium iodide solution (50 µg/ml) was added and analyzed by FACScan cytometry (Becton Dickenson, San Jose, CA).

Immunoblotting (IB, western blotting)

Cells were harvested and homogenized by using the golden lysis buffer. Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories). The protein inputs in the western blot analyses were normalized by loading equal amounts of total protein lysates into the SDS-PAGE gel. Transferred onto polyvinylidene difluoride membranes (PVDF), and then probed with a different primary antibody, followed by secondary antibody conjugated with horseradish peroxidase. Reactive bands were visualized with an enhanced chemiluminescence system (ECL, Amersham).

Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was isolated by using TRIzol reagent (Invitrogen), as recommended by the manufacturer's instructions. The total 2µg RNA was reverse-transcribed into cDNA, using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) 18 primer, by incubating at 37°C for 90 min. Amplification of the cDNA was performed by polymerase chain reaction (PCR) in a final volume of 50 µl containing 2 µl of RT product, dNTPs (each at 200 µM), 1x reaction buffer, a 1 µM concentration of each primer (CDK1, forward 5'- GATTCTATCCCTCTGGT-3', reverse 5'- TAGGCTTCCTGGTTTCC-3'; CDK2, forward

5'- CGCTTCATGGAGAACTTC-3', reverse 5'- ATGG-CAGAAAGCTAGGCC-3'; Cyclin D, forward 5'- CTG TTT GGC GTT TCCCA-3', reverse 5'-T CCT CCT CAC ACC TCC TC3'; GAPDH, forward 5'- TGAAGGTCGGTGTGAACGGATTGGC-3', reverse 5'-CATGTAGGCCATGAGGTC CACCAC-3') and 50 units/ml Pro Taq DNA polymerase. The amplification cycle were 95°C for 30s, 55°C for 1 min and 72°C for 1 min. The sample of each PCR product was separated by electrophoresis on a 1.5% agarose gel after 35 cycles and visualized by ethidium bromide staining.

Statistical analysis

The results obtained are expressed as means $\pm$ S.E. Each value is the mean of at least three separate experiments in each group. The significance of the difference (*p* value) was statistically analyzed by ANOVA, followed by Dunnett's multiple comparison test, to assess the statistical significance (**p* < 0.05).

Results

The combination of Dox and Tam suppressed cell growth and induced morphological changes in breast cancer cells

Combination index (CI) was calculated to assess synergistic effect (CI < 1), additive effect (CI = 1), or antagonistic effect (CI > 1). The CI value of co-treatment with Dox and Tam was evaluated by using MTT assay in estrogen receptor (ER) -positive (BT483), estrogen receptor -negative (MDA-MB-231) and Her2-overexpressing (231eb) breast cancer cells. Dox significantly inhibited the cell proliferation in BT483, MDA-MB-231, 231neo, and 231eB cells in a dose-dependent manner with an IC₅₀ value of 0.67µM, 0.91µM, 1.52µM, and 1.51 µM, respectively (Figure 1A). Similar results were also observed in four breast cancer cell lines treated with Tam. The IC₅₀ value of Tam in four breast cancer cell lines were 6.8µM, 6.1µM, 9.5µM, and 10.5 µM, respectively (Figure 1B). After 48 h of incubation with 0.5µM Dox alone, the number of viable cells in BT483 was 82.3% of the number in the control, whereas the number after incubation with 4µM Tam alone was 98.7%. Combined treatment with 0.5µM Dox and 4µM Tam reduced this number to 21.6% (Figure 1C). The calculation of the CI for Tam and Dox combinations consistently showed CI values < 1, indicating synergism (Table 1). Similar results were also observed in Her2-overexpressing breast cancer cells (231eb). However, co-treatment with Dox and Tam reduced cell proliferation in MDA-MB-231 and 231neo cells only slightly. These findings indicate that Dox and Tam have a synergistic effect on the inhibition of BT483 and 231eb cell growth. The BT483 and 231eb cell lines were selected for further analysis.

The morphological changes in BT483, 231eb and 231neo cells co-treated with different concentrations of Dox and Tam for 48 h are illustrated in (Figure 2). The control cells were tightly attached to the culture plate. However, after combined treatment with Dox (0.5µM) and Tam (4 µM),

Figure 1. Effect of treatment with a combination of Tam and Dox on cell proliferation in breast cancer cells. BT483, MDA-MB-231, 231eB and 231neo cells were cultured in DMEM supplemented with 10% fetal calf serum for 24h. After culturing for 24-h, cells were treated with different concentrations of Dox and Tam for 48h. Cell growth inhibition was determined by MTT assay. The combination of Tam and Dox was more effective than either agent alone: * P < 0.05.

the morphology of the BT483 cells changed dramatically, but not after treatment with either agent alone (**Figure 2A**). Similar results were obtained for 231eb cells (**Figure 2B**).

Dox and Tam synergistically induced cell cycle arrest in BT483 cells

The decreasing number of viable cells may be due to induction of cell cycle arrest or apoptosis. Thus, cell cycle distribution were performed on cells co-treated with Dox and Tam for 48h, using flow cytometry. In BT483 cells, Tam (4 μ M) alone marginally increased the cell population in the G1 phase by 6.8%, as compared to the control, whereas Dox (0.5 μ M) alone drastically increased the cell population in the G2 phase by 35%, as compared to the control. However, treatment with a combination of Dox (0.5 μ M) and Tam (4 μ M) synergistically increased the G2 phase population by 18.0%, as compared to treatment with Tam

Figure 2. Effect of treatment with a combination of Tam and Dox on cell morphology in breast cancer cells. (A) BT483, (B) 231eb, and (C) 231 neo cells were cultured in DMEM supplemented with 10% fetal calf serum for 24h. After culturing for 24-h, cells were treated with different concentrations of Dox and Tam for 48h. Morphological changes in cells were examined by light microscopic observation.

Table 1. Combination index (CI) of co-treatment with Tamoxifen and Doxorubicin.

Cell line	Combination	Combination index (CI)
BT483	0.5 μ M Dox / 4 μ M Tam	0.88
	1 μ M Dox / 4 μ M Tam	1.25
231eB	0.5 μ M Dox / 4 μ M Tam	0.87
	1 μ M Dox / 4 μ M Tam	1.12
231neo	0.5 μ M Dox / 4 μ M Tam	1.54
	1 μ M Dox / 4 μ M Tam	0.71
MDA-MB-231	0.5 μ M Dox / 4 μ M Tam	3.01
	1 μ M Dox / 4 μ M Tam	2.02

CI was defined as follows: $CI = (D_1)/(Dx)_1 + (D_2)/(Dx)_2 + (D_1)(D_2)/(Dx)_1(Dx)_2(Dx)_1$ and $(Dx)_2$ are the concentrations of drug 1 and drug 2, which alone produce x% effect. $(D)_1$ and $(D)_2$ are the concentrations of drug 1 combined with drug 2 to obtain the same response as drug 1 alone or drug 2 alone.

(4 μ M) alone, and increased the G1 phase population by 5.71%, as compared to treatment with Dox (0.5 μ M) alone (**Figure 3A**). These results demonstrate that Dox and Tam act synergistically to arrest breast cancer cells in G1 and G2 phase.

Figure 3. Changes in cell cycle phase distribution following Tam and Dox treatment.

(A) BT483 (B) MDA-MB-231 (C) 231eB (D) 231neo cells were cultured in DMEM supplemented with 10% fetal calf serum for 24h culture. After culturing for 24-h, cells were treated with different concentrations of Dox and Tam for 48h. Cells were harvested and stained with propidium iodide and the cell cycle distribution was analyzed by flow cytometry.

The combination of Dox and Tam modulated expression of cell cycle regulatory proteins in BT483 breast cancer cells

To identify the underlying molecular mechanism of the synergy between Dox and Tam, a western blotting analysis was used to profile the expression of cell cycle regulatory proteins in BT483 cells treated with a single agent and combination therapy. (Figure 4A and 4B) shows that Dox and Tam alone, or in combination, do not decrease cyclin A, cyclin B, cyclin E, cdc25, cullin 1, p21, or p27, erb2, β -Trcp protein level. However, co-treatment with Dox and Tam decreases CDK1, CDK2 and cyclin D in BT483 cells. The results show that the synergistic action of a combination of Dox and Tam on G1 and G2 phase cell cycle arrest in BT483 cells occurs because of their synergistic action on the down-regulation of CDK1, CDK2, and cyclin D. In order to identify the mechanism in the culture co-treated with Dox and Tam decrease the protein level of CDK1, CDK2 and cyclin D, the mRNA level of CDK1, CDK2 and cyclin D was first examined. As shown in (Figure 4C), no significant

Figure 4. Effects of dose and treatment time for treatment with a combination of Tam and Dox on expression levels of cell cycle regulatory proteins in BT483 cells.

BT483 cells were treated with DMSO alone (CON), 4μM Tam, 4μM Tam plus varying concentrations of Dox, for 48h (A), or 4μM Tam plus 0.5μM Dox for the indicated times (24h, 48h) (B). Total cell lysates were prepared and western blot analysis was performed. β -Actin was used as an internal control, to ensure equivalent protein loading. (C) BT483 cells were treated with DMSO alone (CON), 4μM Tam, 4μM Tam, plus various concentrations of Dox for 48h. Total RNA was isolated and the mRNA expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

change in CDK1 and CDK2 levels is observed in the culture co-treated with Dox and Tam. These results indicate that

the decrease in CDK1 and CDK2 protein levels produced by treatment with Dox and Tam involves a posttranscriptional mechanism. In contrast, combined treatment with Dox and Tam synergistically decreases cyclin D mRNA expression in BT483. These results indicate that the decrease in cyclin D protein levels produced by cotreatment with Dox and Tam involves a transcriptional mechanism. Further experiments would be required to determine this possibility.

Growth inhibition of BT483 by CDK1/2 inhibitor NU6102

Combined treatment with Dox and Tam decreases CDK1 and CDK2 expression and causes cell cycle arrest in BT483 cells. This study investigates whether this can be mimicked using small-molecule CDK1/2 inhibitors, NU6102. In (Figure 5A), 5μM NU6102 decreased cell growth, cyclin A, CDK1 and CDK4 protein levels, but increased p21 and p27

protein levels. Taken together, these results suggest that NU6102 decreases cell growth by repressing CDK2 protein expression in BT483 cells.

Combined treatment with Dox and Tam modulated the phosphorylation of intracellular signaling proteins
AKT, ERK, MAPKs and PKC have been shown to be involved in CDK1, CDK2 and cyclin D regulation in various cell types [13-17]. In order to examine whether the activities of AKT, ERK, MAPKs and PKC are affected by combined treatment with Dox and Tam, the phosphorylation of Akt, ERK, MAPKs and PKC in BT483 cells was analyzed after combined treatment with Dox and Tam for 12 h and 24 h. In (Figure 6), combined treatment with Dox and Tam synergistically increases ERK phosphorylation. 0.5μM Dox treatment inhibits the phosphorylation of Akt, but combined treatment with Dox and Tam does not produce any synergistic effect on AKT activity. These results suggest that ERK were activated and played important roles in BT483 cells co-treated with Dox and Tam.

Discussion

Several studies have already reported that a combination of adjuvant endocrine therapy and chemotherapy is better than either treatment alone. These two therapeutic approaches can be combined without a significant increase in toxicity. Additionally, because their mechanisms are presumed to be different, there is a potential for an additive or synergistic effect. Although combining adjuvant endocrine therapy, using Tam, and chemotherapy, using Dox, has previously been studied in various clinical settings, the mechanism has not been established. This study is the first report to show that combined treatment with Dox and Tam has an enhanced effect on their inhibition of proliferation through down-regulation of CDK1, CDK2 and cyclin D expression

in BT483 cells. This increased efficacy might also allow a reduction of Tam or Dox dosage, to minimize the negative side effects.

Combinations of chemotherapy and Tam adjuvant have yielded conflicting results for overall survival. Most studies report a benefit for Tam in receptor positive patients [9,18,19], while two trials found evidence of benefit in receptor negative patients [20,21]. Some trials have also reported statistically insignificant beneficial effects for the addition of Tam to adjuvant chemotherapy [22,23]. Ahmann *et al.*, reported that combined chemohormonal therapy with Tam, Dox and cyclophosphamide resulted in a higher objective response rate and a long median survival in patients with ER-positive tumors [24]. Similarly, another study found that the combination of radiotherapy, Dox and Tam seemed to improve both relapse-free and overall survival in ER-positive tumors [5]. The results of this study tend to support the findings of the two earlier with respect to the synergistic effect on cell survival in ER-positive breast cancers, but not in ER-negative breast cancers.

Conclusions

In summary, it has been demonstrated that the combination of Dox and Tam significantly reduces the growth of ER-positive breast cancer cells and that this is driven primarily by the enhanced effect of the decreased protein expression of CDK1, CDK2 and cyclin D. It is also proposed that selective modification of AKT inactivation and ERK activation probably contributes to the synergistic interaction. Overall, these findings suggest that a combination of Dox and Tam could be effective for the treatment of ER-positive breast cancers, so this combination warrants further investigation.

Competing interests

The authors declare that they have no competing interests.

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