In vitro antiproliferative effects of nab-paclitaxel with liposomal cisplatin on MDA-MB-231 and MCF-7 breast cancer cell lines
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Summary

**Purpose:** In this study, the in vitro cytotoxic effect of nanotechnological drugs nab-paclitaxel and liposomal cisplatin combination was evaluated on MDA-MB-231 and MCF-7 breast cancer cell lines.

**Methods:** For this purpose cell viability, cell index values obtained from xCELLigence RTCA (Real-Time Cell Analysis) DP instrument, mitotic index (MI), apoptotic index (AI) and labelling index (LI) analysis among cell kinetic parameters were used. A1L25: 1 µg/ml nab-paclitaxel+25 µg/ml liposomal cisplatin, A1L5: 1 µg/ml nab-paclitaxel+5 µg/ml liposomal cisplatin and A10L5: 10 µg/ml nab-paclitaxel+5 µg/ml liposomal cisplatin for MDA-MB-231 cell line and A1L5: 1 µg/ml nab-paclitaxel+5 µg/ml liposomal cisplatin, A1L10: 1 µg/ml nab-paclitaxel+10 µg/ml liposomal cisplatin and A5L1: 5 µg/ml nab-paclitaxel+1 µg/ml liposomal cisplatin doses for MCF-7 were applied for 24-72 hrs.

**Results:** Significant decrease in cell viability and cell index values for both cell lines was observed, while the MI and LI values of both cell lines increased at 24 hrs, and decreased significantly at 72 hrs. Also there was a significant increase in the AI values.

**Conclusions:** Nab-paclitaxel and liposomal cisplatin offer a promising treatment modality in different breast cancer subtypes.

**Key words:** liposomal cisplatin, MCF-7, MDA-MB-231, Nab-paclitaxel, xCELLigence RTCA (Real-Time Cell Analysis) DP instrument

Introduction

Combination therapy is the simultaneous administration of two or more pharmacologically active agents with different mechanisms. Combination therapy has long been adopted as a primary cancer treatment [1]. Compared with single-drug therapy, combined therapy is capable of reducing drug resistance by targeting different signal pathways [2].

Different cell cycle specificity, different mechanism of action, effectiveness as single agent, different side effects and different mechanisms of resistance development are the main criteria for selecting a combination therapy [3].

Two commonly utilized chemotherapeutics in cancer treatment are cisplatin and paclitaxel [4]. Paclitaxel enhances tubulin polymerization to stable microtubules and stabilizes them against depolymerization, which results in mitotic arrest [5]. Cisplatin inhibits cell proliferation through multiple mechanisms, including binding with DNA to form intra-strand adducts causing changes in DNA conformation, promoting mitochondrial damage leading to diminished energy production, altering cellular transport mechanisms, and decreasing ATPase activity within the cells [6,7].

Nab-paclitaxel and liposomal cisplatin are the nanotechnological formulations of paclitaxel and cisplatin. Nab-paclitaxel, an albumin-bound 130-
nm particle form of paclitaxel, is solvent-free and was designed to improve the therapeutic index of paclitaxel, i.e. to increase antitumor activity and reduce toxicities associated with the cremophor such as hypersensitivity reactions. Compared with solvent based-paclitaxel, nab-paclitaxel has demonstrated enhanced transport across endothelial cell monolayers and greater tumor delivery of paclitaxel in preclinical models [8]. 110 nm particle liposomal cisplatin is a cisplatin-liposome formulation with the liposome consisting of dipalmityl phosphatidyl glycerol, soy phosphatidyl choline, cholesterol and methoxy-polyethylene glycol-distearoyl phosphatidyl phosphatidyl-ethanolamine [9]. The liposomes display preferential tumor uptake compared to surrounding non-cancerous tissues due to the EPR (Enhanced permeability and retention) effect [10]. Liposomal cisplatin also shows no nephrotoxicity and lacks the serious side effects of cisplatin, while seeming to retain the efficacy of cisplatin [9,11].

In this study, we investigated the antiproliferative effects of nab-paclitaxel and liposomal cisplatin together on MCF-7 cell line which belongs to Luminal A subtype of breast cancer and MDA-MB-231 cell line which belongs to basal subtype of breast cancer.

Methods

Cell culture

The MDA-MB-231 estrogen receptor-negative cells derived from a metastatic carcinoma, and the human breast epithelial cell line MCF-7, estrogen receptor-positive cells derived from an in situ carcinoma used in this study were obtained from European Cell Culture Collection (CCL). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (high glucose) (Gibco Lab) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 μg/ml streptomycin (streptomycin sulphate, I. E. Ulugay, Istanbul, Turkey), 100 IU/ml penicillin (Pronapen, Pfizer, Istanbul, Turkey), amphotericin B (Sigma, USA) and 2 mM glutamine at 37°C in humidified atmosphere of 5% CO₂. The pH of the medium was adjusted to 7.4 with NaHCO₃.

Drug doses

In this study different combinations of nab-paclitaxel and liposomal cisplatin were determined. For MDA-MB-231 cell line A1L25: 1 μg/ml nab-paclitaxel +25 μg/ml liposomal cisplatin, A1L5: 1 μg/ml nab-paclitaxel+5 μg/ml liposomal cisplatin and A10L5: 10 μg/ml nab-paclitaxel+5 μg/ml liposomal cisplatin doses and for MCF-7 cell line A1L5: 1 μg/ml nab-paclitaxel+5 μg/ml liposomal cisplatin, A1L10: 1 μg/ml nab-paclitaxel+10 μg/ml liposomal cisplatin and A5L1: 5 μg/ml nab-paclitaxel+1 μg/ml liposomal cisplatin doses were used.

Preparing ³H-thymidine

Nine ml of deionized water were added to a vial containing 1 mCi/ml ³H-thymidine (TRA-120, Amerham, England) and stock solution was prepared. Then 1 mCi/ml solution was diluted to 1 μCi/ml with cell culture medium for the cells to be labelled with this solution.

Cell viability analysis

Cell viability was examined using the MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma, Missouri, USA) colorimetric assay. Briefly, MDA-MB-231 and MCF-7 cells were plated in 96-well plates at a density of 2x10⁴ cells per well and each plate was incubated for 24 hrs. After incubation, drug doses were added to each well. At the end of the experimental period, the medium in each well was removed and 40 μl fresh MTT solution (5 mg/ml in PBS) were added into each well and cells were incubated at 37 °C for 4 hrs. Then, DMSO (Dimethyl Sulfoxide, Sigma, France) was added into each well and cells were shaken thoroughly for 1 hr on a shaker. Then, the absorbance of the samples was measured against a background control as a blank using an Elisa reader (μQuant, Bio-Tek Instruments Inc Vermont, USA) at 450-690 nm.

xCELLigence Real-Time Cell Analysis (RTCA): cytotoxicity

Experiments were carried out using the xCELLigence RTCA DP instrument (Roche Diagnostics GmbH, Mannheim, Germany) which was placed in a humidified incubator at 37°C and 5% CO₂. Cytotoxicity experiments were performed using modified 16-well plates (E-plate, Roche Diagnostics GmbH, Mannheim, Germany). Microelectrodes were attached at the bottom of the wells for impedance-based detection of attachment, spreading and proliferation of the cells. The background impedance signal was measured with 100 μl of cell culture medium/well. The final volume in a single well was adjusted to 200 μl of cell culture medium by adding an additional 100 μl of medium containing cells. Cell numbers were 5000 cell/well for MDA-MB-231 and 10000 cell/well for MCF-7. The impedance was recorded in 15 min intervals. Twenty hrs after seeding, drug concentrations were added to the culture. All incubations were performed at a volume of 200 μl.

Mitotic index (MI)

MI was determined by the Feulgen method. Before the cells were treated with Feulgen, they were treated with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min at 60°C. After slides were treated with Feulgen, they were rinsed for a
few min in distilled water and stained with 10% Giemsa stain solution (pH 6.8) for 5 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water, and then the slides were air-dried. At the end the MI was calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control and at least 3,000 cells were examined from each slide for MI.

**Apoptotic index (AI)**

The AI (the percentage of cells undergoing apoptosis) was studied using fluorescence microscope. For the determination of the AI, cells were fixed with methanol and stained with 4′-6 diamidine-2 phenylindol (DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored under fluorescence microscope. For evaluation of the AI, at least 100 cells were counted for control and each of the experimental groups.

**3H-thymidine labelling index analysis**

For 3H-thymidine labelling index analysis, which determines cells in the S phase, MDA-MB-231 and MCF-7 cells were seeded into round coverslips which were in 24-well plates at a density of 2x10⁴ cells per well and incubated 24 hrs. Then the cells were treated with the combined doses of nab-paclitaxel and liposomal cisplatin. At the end of the experimental period, cells were treated with medium containing 1 μCi/mL 3H-thymidine for 20 min to evaluate the labelling index.

**Autoradiography**

After labelling, the cells were fixed with Carnoy’s fixative (3:1 methanol-acetic acid) and the remaining radioactive material was washed twice with 2% perchloric acid at 4°C for 30 min. Coverslips were prepared and were coated with K.2 gel emulsion (Ilford, Cheshire, UK) prepared with distilled water at 40°C to determine thymidine labelling index. After 3-days exposure at 4°C, autoradiograms were washed with D-19 b developer (Kodak, New York, USA) and fixed with Fixaj B (Kodak, New York, USA). The coverslips were evaluated after being stained with Giemsa for 3 min. The labelled cells were counted on each coverslip. At least 3,000 cells were examined from each coverslip.

**Statistics**

Values of cell viability, MI, AI and LI were evaluated relative to controls and to each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT’s test and the significance between experimental groups was determined by Student’s t-test. A p value < 0.01 was accepted as statistically significant.

**Results**

**Determination of optimal dose with cell viability analysis**

The absorbance values were 65.57x10⁻³; 53.42x10⁻³; 32.45x10⁻³ and 28.51x10⁻³ respectively for control, and A1L25, A1L5, A10L5 for MDA-MB-231 cell line for 24 hrs (Figure 1). The results indicated that 24 hrs after the administration of combined doses to MDA-MB-231 cells viability values were 81.47% for A1L25, 49.48 for A1L5 and 43.48 for A10L5 compared to the control group which was considered as 100% (Figure 2). The differences between control and all experimental groups were statistically significant (p<0.01).

**Figure 1.** Absorbance values of mitochondrial dehydrogenase activity (450-690 nm) of MDA-MB-231 cells treated with A1L25, A1L5 and A10L5 doses of nab-paclitaxel and liposomal cisplatin (A1L25: 1 μg/ml nab-paclitaxel + 25 μg/ml liposomal cisplatin, A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin and A10L5: 10 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin) for 24 hrs (p<0.01).

**Figure 2.** Percent viability values of MDA-MB-231 cells treated with A1L25, A1L5 and A10L5 doses of nab-paclitaxel and liposomal cisplatin (A1L25: 1 μg/ml nab-paclitaxel + 25 μg/ml liposomal cisplatin, A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin and A10L5: 10 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin) for 24 hrs (p<0.01).
The absorbance values were 335.27x10^{-3}, 167.18x10^{-3}, 143.14x10^{-3} and 108.43x10^{-3} respectively for the controls, A1L5, A1L10 and A5L1 for MCF-7 cell line for 24 hrs (p<0.01).

The absorbance values were 355.27x10^{-3}, 167.18x10^{-3}, 143.14x10^{-3} and 108.45x10^{-3} respectively for the controls, A1L5, A1L10, A5L1 for MCF-7 cell line for 24 hrs (Figure 3). The results indicated that 24 hrs after the administration of combined doses to MCF-7 cells viability values were 49.86% for A1L5, 42.69 for A1L10 and 32.34 for A5L1 compared to control group which was considered as 100% (Figure 4). The differences between control and all experimental groups were statistically significant (p<0.01).

Cell viability

As seen in Figure 5, after administration of A1L25, A1L5 and A10L5 drug concentrations for 0-72 hrs the cell proliferation values of MDA-MB-231 cells and as seen in Figure 6, after administration of A1L5, A5L1, A1L10 drug concentrations for 0-72 hrs, the cell proliferation values of MCF-7 cells decreased significantly, especially at 48 and 72 hrs. The differences between the control and all experimental groups were significant (p<0.01).

xCELLigence Real-Time Cell Analysis (RTCA): cytotoxicity

Cell index values obtained from xCelligence real-time cell analysis system showed that the combination of nab-paclitaxel and liposomal cisplatin had significant antiproliferative effects on both MDA-MB-231 and MCF-7 cell lines. These values also showed that while combination doses had antimitotic effects on cells as a result of application of A1L25, A1L5 and A10L5 doses of the
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**Figure 7.** Cell index values of MDA-MB-231 cells treated with A1L25, A1L5 and A10L5 doses of nab-paclitaxel and liposomal cisplatin (A1L25: 1 μg/ml nab-paclitaxel + 25 μg/ml liposomal cisplatin, A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin and A10L5: 10 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin) obtained from xCelligence Real-Time Cell Analysis (RTCA) system (Line A: Control, Line B: A1L25, Line C: A1L5, Line D: A10L5).

**Figure 8.** Cell index values of MCF-7 cells treated with A1L5, A5L1, A1L10 doses of nab-paclitaxel and liposomal cisplatin (A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin, A1L10: 1 μg/ml nab-paclitaxel + 10 μg/ml liposomal cisplatin and A5L1: 5 μg/ml nab-paclitaxel + 1 μg/ml liposomal cisplatin) obtained from xCelligence Real-Time Cell Analysis (RTCA) system (Line A: Control, Line B: A1L5, Line C: A1L10, Line D: A5L1).

**Figure 9.** Mitotic index values of MDA-MB-231 cells treated with A1L25, A1L5 and A10L5 doses of nab-paclitaxel and liposomal cisplatin (A1L25: 1 μg/ml nab-paclitaxel + 25 μg/ml liposomal cisplatin, A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin and A10L5: 10 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin) for 0-72 hrs (p<0.01).

**Figure 10.** Mitotic index values of MCF-7 cells treated with A1L5, A1L10 and A5L1 doses of nab-paclitaxel and liposomal cisplatin (A1L5: 1 μg/ml nab-paclitaxel + 5 μg/ml liposomal cisplatin, A1L10: 1 μg/ml nab-paclitaxel + 10 μg/ml liposomal cisplatin and A5L1: 5 μg/ml nab-paclitaxel + 1 μg/ml liposomal cisplatin) for 0-72 hrs (p<0.01).

drugs on MDA-MB-231 for 72 hrs (Figure 7), combination doses had DNA damaging effects on cells as a result of application of A1L5, A5L1, A1L10 doses of the drugs on MCF-7 for 72 hrs (Figure 8).

**Mitotic index (MI)**

As seen in Figures 9 and 10, after administration of A1L25, A1L5 and A10L5 doses on MDA-MB-231 cell line for 0-72 hrs and A1L5, A5L1, A1L10 doses on MCF-7 cell line for 0-72 hrs, while the MI values of both of cell lines increased at 24 hrs, they decreased significantly at 72 hrs. The differences between the control and all experimental groups were significant (p<0.01).

**Labelling index (LI)**

As seen in Figures 11 and 12, after administration of A1L25, A1L5 and A10L5 doses on MDA-MB-231 cell line for 0-72 hrs and A1L5, A5L1, A1L10 doses on MCF-7 cell line for 0-72 hrs, while the labelling index values of both of cell lines increased at 24 hrs, they decreased significantly at 72 hrs. The differences between the control and all experimental groups were significant (p<0.01).

**Apoptotic index (AI)**

As seen in Figures 13 and 14, after administration of A1L25, A1L5 and A10L5 drug concentrations on MDA-MB-231 cell line and A1L5, A5L1, A1L10 doses on MCF-7 cell line for 0-72 hrs, the AI values of both cell lines increased significantly in a time-dependent manner. The differences between the control and all experimental groups were significant (p<0.01).
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Discussion

One of the most critical points in breast cancer treatment is early stage diagnosis, before tumor cells metastasize. In addition, current diagnostic and therapeutic approaches rely predominantly on conventional methods [12,13]. Unfortunately, cancer therapies are limited by inadequate drug concentrations reaching the tumor. The rapid elimination and widespread distribution of the drug into targeted organs and tissues requires its administration in large quantities which often result in systemic toxicity and adverse effects [14,15].

The goal of this study was to evaluate the cytotoxic effects of nab-paclitaxel and liposomal cisplatin combination on estrogen receptor-negative MDA-MB-231 cells derived from a metastatic carcinoma, and the human breast epithelial cell line estrogen receptor-positive MCF-7 cells derived from an in situ carcinoma. For this purpose various cell kinetic parameters including cell index values obtained from xCELLigence RTCA (Real-Time Cell Analysis) DP instrument, MI, AI and LI analysis were used.

Antimitotic agents are used extensively in cancer chemotherapy. These agents target microtubules and thus block mitotic progression by activating the spindle assembly checkpoint. Following a prolonged mitotic arrest, cells either die in mitosis via apoptosis, or exit mitosis without dividing and survive, a process known as slippage [16]. These type of drugs enhance microtubule polymerization and promote microtubule assembly [17,18], which blocks transit of cells through the G, and M phases of the cell cycle [17,19]. It
has also been observed that paclitaxel induces nuclear fragmentation in cultured cells [20], a feature characteristic of apoptotic cell death [21]. The nanotechnological formulation of paclitaxel, nab-paclitaxel, displays the same effect.

Mitosis and apoptosis display several similar morphological features. Both mitotic and apoptotic cells lose substrate attachment and become rounded. During both processes, cells shrink, condense their chromatin, and display rapid membrane blebbing. Although a number of similarities exist between mitotic and apoptotic cells, several distinct differences are also apparent [22].

Cisplatin is used for the treatment of a wide variety of solid malignancies and exerts anticancer effects via multiple mechanisms [23]. Cisplatin is a DNA cross linking agent that interferes with mitosis and triggers apoptosis or cell death. Its effectiveness lies in how easily it releases its platinum molecule to cross link DNA strands, which in turn disrupts cell division [24]. The nanotechnological form of cisplatin, liposomal cisplatin, is one of the most promising liposomal platinum drug formulations under clinical investigation [25].

As previously demonstrated in cervical cancer, liposomal cisplatin exerted its cytotoxic effect by inducing apoptosis [26]. Casagrande et al. have shown that liposomal cisplatin induced apoptosis and reactive oxygen species (ROS) production, reduced spheroid growth and migration, and reduced cancer stem cell (CSC) number. Liposomal cisplatin inhibited xenograft tumor growth to more than 90% and with low toxicity, whereas the effective dose of cisplatin was too toxic for the animals. Liposomal cisplatin showed a synergistic activity with doxorubicin and nab-paclitaxel [25].

Our results showed that there was a significant decrease in cell viability and cell index values for both MDA-MB-231 and MCF-7 cell lines, while the MI and LI values of both of cell lines increased at 24 hrs, and decreased significantly at 72 hrs. Also there was a significant increase in apoptotic index values. These results are consistent with the studies mentioned above. In conclusion, replacing paclitaxel with nab-paclitaxel and cisplatin with liposomal cisplatin in different types of breast cancer adds the advantage of lower toxicities as already shown in the current conventional therapy. Combination of these drugs increases the treatment efficacy.

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Conflict of interests

The authors declare no conflict of interests.

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