·论　著·

In Vitro Evaluation of Schedule - dependent Interaction between Docetaxel and Cisplatin against Human Breast Cancer Cells[1]

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ABSTRACT　OBJECTIVE: To study the schedule dependent interactions between docetaxel and cisplatin against human breast cancer cells. **METHOD:** The assays in vitro were used including DNA fragmentation analyse, MTT assay, flow cytometric analyses, and western blotting. **RESULTS:** The antagonistic interaction occurred when tumor cells were exposed to the two drugs simultaneously or exposed to cisplatin before docetaxel. No antagonism was observed when docetaxel was added before cisplatin. **CONCLUSIONS:** Cisplatin could interfere with the cytotoxic effects of docetaxel on both mitotic arrest and apoptosis. In addition, the combination of two drugs could inhibit the bcl-2 phosphorylation that induced by docetaxel. These results indicate that interaction between docetaxel and cisplatin is highly schedule dependent.

KEY WORDS　Docetaxel, Cisplatin, Schedule-dependent Interaction, BCap37 cell line

体外评估多烯紫杉醇与顺铂给药顺序依赖性的相互作用[1]

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摘要　目的:将多烯紫杉醇与顺铂以不同给药顺序处理人乳腺癌细胞研究两者细胞毒性的相互作用。方法:应用一系列体外实验如 DNA 片段分析、MTT 测定、流式细胞仪分析和蛋白印迹法分析两者以不同给药顺序的实验结果。结果:显示当同时给药或顺铂在多烯紫杉醇前给药则发生拮抗作用;多烯紫杉醇在顺铂前给药则不发生拮抗作用。结论:顺铂干扰多烯紫杉醇对癌细胞的捕获和细胞凋亡,并影响多烯紫杉醇诱导的 bcl-2 磷酸化;多烯紫杉醇与顺铂的相互作用具有高度给药顺序依赖性。

关键词　多烯紫杉醇,顺铂,给药顺序依赖性,BCap37 细胞系

Introduction

Docetaxel [Taxotere, N-debenzol-N-tert-butoxycarbonyl-10-deacetyltaxel(Fig1)], a novel member of the taxoid family, was prepared by a semi-synthesis approach from 10-deacetyl baccatin Ⅲ, an inactive precursor extracted from the needles of the European yew Taxus baccata that is condensed with the fully synthetic side chain in 1986. Due to this efficient process using a renewable source of natural precursor (needle instead of bark), realistic possibilities for the pharmaceutical development of taxoids were opened. Docetaxel is a mitotic spindle poison that exerts its anticancer effects by promoting the assembly of microtubules and inhibiting their depolymerization. Cells are thus arrested in the S and G2/M-phase and are unable to divide. In preclinical and clinical studies, Docetaxel has demonstrated activity against a variety of tumor types, including breast, ovarian, non-small cell lung, head and neck cancers[1].

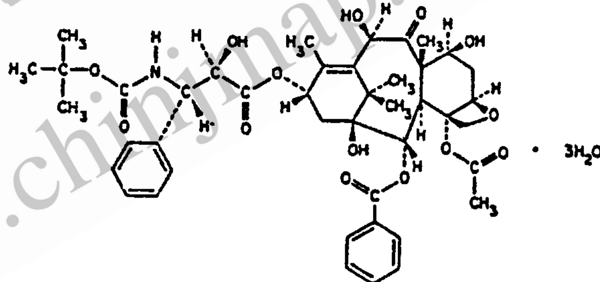

Fig 1.　Chemical structure of docetaxel

Cisplain [cis-platinum(II)-diammine Dichlodide] is one of the most commonly used anticancer agent, with documented activity against a number of solid tumors, notably of the testic, ovaries

[1] This project was supported by NIH Grants CA71851 and CA82440 (to Fan W.)

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and bladder. Although it is not widely used in the treatment of breast cancer, cisplatin has achieved response rates of about 50% in patients with previously untreated metastatic disease. However, the use of cisplatin is limited by severe toxicities, including nephrotoxity, myelosuppression, ototoxity and neurotoxity. The use of low doses of cisplatin in combination with agents that do not have overlapping toxicity may thus optimize the benefit achievable with this drug. The feasibility of combining docetaxel with cisplatin in patients with solid tumors and advanced non-small cell lung cancer has been demonstrated in phase I and II studies[2].

The experimental data on combination in vitro are limited for docetaxel and other anticancer agent. It has demonstrated that 5-FU and glucocorticoids interact with paclitaxel, one of taxoids, using different cell lines[3,4]. In order to clarify this issue, we investigated the interaction between docetaxel and cisplatin using human breast tumor BCap37 cell lines to elucidate the possible mechanism of interactions and provide the basis for promoted clinical therapeutic benefit in combination chemotherapy.

Materials and Methods

Drugs and cell culture. Docetaxel was purchased from Rhone-Poulenc Inc.(Antony, France) and dissolved in dimethyl sulfoxide(DMSO) to make a stock solution of 1.0mmol/L. Cisplatin was obtained from Sigma Chemical Co. (MO, USA) and dissolved in DMSO to make a stock solution of 1.0 mmol/L. The stock solutions were diluted in culture medium to obtain the desired concentrations. The human breast tumor BCap37 cell line was propagated in RPMI 1640 medium supplemented with 10% heat-inactive newborn calf serum (Life technologies Inc, NY USA) and 1% antibiotic-antimycotic (Life technologies Inc, NY USA). MTT{3-[4,5-dimethyl(-thiazol-2-zyl)-2,5-diphenyltetrozolium bromide]}, propidium iodide and ethidium bromide were purchased from Sigma Chemical Co.(St. Lowis, MO, USA). Other reagents and chemicals were obtained from commercial sources. The cells were usually treated with drug when they reached approximately 60-70% confluence.

Determination of DNA fragmentation. Internucleosomal DNA fragmentation was assayed by a modification of previously described methods[5]. Briefly, cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS) once. Cells were suspended in lysis solution containing 20mmol/L Tris-HCl, 5 mmol/L EDTA and 5%(V/V) Triton X-100. The crude DNA samples were extracted with the solution of Tris saturated phenol chloroform. After phase separation, 50μl of 5 mol/L NaCl and 0.5 ml isopropanol were added into the supernatant. The DNA pellet was washed with 1 ml of 70% prechilled ethanol and then dried under vacuum. The DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2μg/ml ethidium bromide and visualized under UV illumination (Transilluminator 2020E UV/WHITE equipped with CCD 100 DAGE-MIT Digital Camera and Workstation with Snap Software).

Flow Cytometric Analysis. The cell sample preparation and propidium iodide (PI) staining were performed according to the previously reported method[6]. Briefly, cells were incubated with 5nmol/L docetaxel or 1.0μmol/L cisplatin or their combination for 24h and 48h. Cells were harvested by trypsinization at the times indicated and washed twice with prechilled PBS. Cells were fixed in 1% formaldehyde in PBS and then dehydrated in 80% ethanol in prechilled PBS. Approximately 45 min before immunoflow cytometry analysis, cells were incubated in PBS containing 0.1 mg/ml RNase A and 40μg/ml PI at room temperature and kept in dark. Cell cycle distribution was determined using a Coulter Epics V Instrument (Coulter Corp) with an argon laser set to excite wavelength at 488nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA).

MTT Assays. Cells were harvested with trypsin and resuspended to a final concentration of 2×10^4 cells/ml in fresh medium. Aliquots of 0.2ml from each suspension were distributed evenly into 96-well tissue culture plates with lids (Falcon, Oxnard, CA). Designated columns were treated with 5nmol/L docetaxel or 1.0μmol/L cisplatin or their combination. One column from each plate contained medium alone, and another column contained cells without drug as a blank control. After cells were incubated at 37 ℃for 24 h, 0.1 ml of MTT(1 mg/ml) solution was added to each well. After 3h incubation at 37 ℃ to allow viable cells to reduce the yellow MTT into dark blue formosan crystals, the resulting crystals were dissolved in 100μl of DMSO. The absorbance in individual wells was determined for each drug alone and for various drug combinations.

Western Blotting. Cells treated with docetaxel, Cisplatin or their combinations at different schedules were harvested by trypsinization at the time indicated. Briefly, cells were washed twice with cold PBS and cellular protein samples were extracted with buffer containing 10nmol/L Tris, 5nmol/L EDTA, 150mmol/L NaCl, 0.1% Triton X-100(v/v) and 5%glycerol(v/v) and 2% SDS in deionization water. Protein contents in samples were measured using Bio-Rad Protein Assay. Protein samples were loaded onto a 12%SDS polyacrylamide gel at equal protein concentration. After electrophoresis, samples were transferred to a nitrocellulose membrane according to the Bio-Rad protocol. Primary antibody against bcl-2 was used at 1:1000 dilution in 5% no fat milk in TBST(PBS containing 0.5% tween 20). The secondary antibody goat antimouse IgG conjugated to

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horseradish peroxidase at a concentration of 0.1μg/ml in 5% no fat milk in TBST. The immunoreactive bands were visualized using a chemiluminescent substrate to horseradish peroxidase and exposure to Kodak X-OMAT film.

Results

Cisplatin affects docetaxel-induced apoptotic cell death. To investigate the possible influence of cisplatin on docetaxel induced apoptotic cell death, we examined whether docetaxel-induced DNA fragmentation would be affected by cisplatin or their co-exposure. The characteristic DNA fragmentation ladders on agarose gel electrophoresis were observed following exposure to 5nmol/L docetaxel alone for 48h. When cells were treated with 1.0μmol/L or 5.0μmol/L cisplatin alone, there were no DNA fragmentation ladders. When cells were treated with cisplatin first then docetaxel, DNA fragmentation ladders were very weak. When cells were treated with docetaxel first then cisplatin, the DNA fragmentation ladders induced by docetaxel were not affected by cisplatin at both concentrations used (Fig. 2). When cells were treated with docetaxel and cisplatin simultaneously, clear DNA fragmentation ladders were observed.

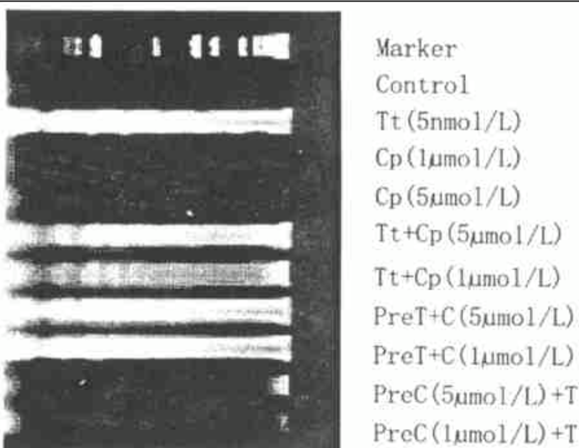

Fig 2. Characterization of effecting of cisplatin on docetaxel-induced DNA fragmentation in BCap37 cells. Tt, docetaxel; Cp, cisplatin; Tt + Cp, docetaxel and cisplatin were added simultaneously; PreC + T, cisplatin was added 24h before docetaxel; PreT + C, docetaxel was added 24h before cisplatin.

Flow cytometric analysis. We assessed changes in the cell cycle distribution after BCap37 cells were exposed to 5nmol/L docetaxel or 1.0μmol/L cisplatin or their combination for 24h or 48h, respectively. The influence of drugs on cell cycle distribution was shown in Fig3. Docetaxel produced an accumulation of cells in S phase. The majority of cells exhibited Gl phase DNA content 24h after treatment with cisplatin. When cells were exposed to docetaxel and cisplatin simultaneously, the cells went to apoptosis at 24 and 48h. If cells were pre-exposed to docetaxel then cisplatin, the cells were arrested in S phase at 24h and went to apoptosis at 48h. When cells pre-exposed to cisplatin then docetaxel, the cells were arrested in G2/M phase at 24h.

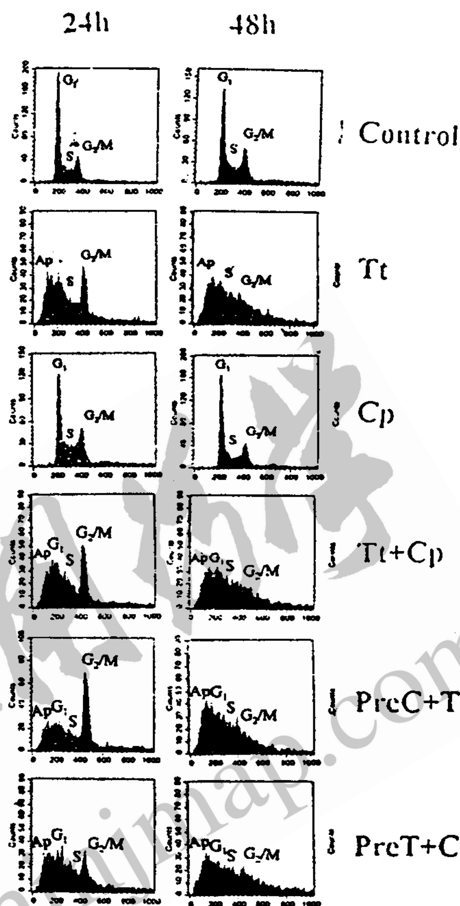

Fig 3. Flow cytometric analyses of BCap37 cells treated with docetaxel, cisplatin, or their combination for 24 or 48h. Tt, docetaxel; Cp, cisplatin; Tt + Cp, docetaxel and cisplatin were added simultaneously; PreC + T, cisplatin was added 12h before docetaxel; PreT + C, docetaxel was added 12h before cisplatin.

Schedule-dependent antagonistic effect of cisplatin on the cytotoxicity of docetaxel. To investigate the possible influence of cisplatin on the antitumor activity of docetaxel, we first used MTT assays to assess the cytotoxic interaction of these two drugs in BCap37 cells. It was examined by treatment of cells with 5nmol/L docetaxel or 1.0μmol/L cisplatin or their combination at various schedules for 24h and 48h. The results, which were summarized in Fig4, showed that pretreatment or simultaneous treatment of cells with 1.0μmol/L cisplatin and docetaxel produced less cytotoxic effects than treatment with docetaxel alone. However an increased cytotoxicity was observed when cells were exposed to docetaxel for 12h before the addition of cisplatin. These results suggested that pretreatment or simultaneous treatment with cisplatin might antagonize the cytotoxic effect of doc-

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etaxel.

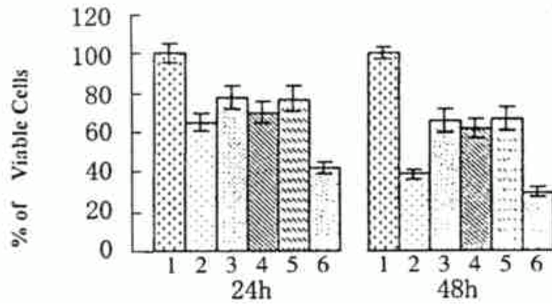

Fig 4. Cytotoxic effects of sequential exposure to docetaxel and cisplatin in BCap37 cells. 1. Control 2. docetaxel; 3. cisplatin; 4. docetaxel and cisplatin were added simultaneously; 5. cisplatin was added 12h before docetaxel; 6. docetaxel was added 12h before cisplatin.

Effects on phosphorylation of bcl-2. To investigate whether docetaxel can modulate apoptosis or cell cycle regulatory proteins, western blotting was performed to analyze the possible alteration of protein in BCap37 cells treated with docetaxel, cisplatin or their combination in different schedules. The results depicted in Fig 5. showed that docetaxel was found to induce phosphorylation of bcl-2. cisplatin alone did not alter bcl-2 protein, but when cells were exposed to docetaxel and cisplatin simultaneously or exposed to cisplatin first for 12h, the ability of docetaxel to induce bcl-2 phosphorylation was inhibited.

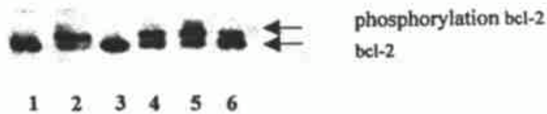

Fig 5. Western blot analysis for bcl-2 protein extracted from BCap37 cells treated with docetaxel, cisplatin, or their combination. 1: Control 2: 5nmol/L Docetaxel 3: 1μmol/L Cisplatin 4: 5nmol/L Docetaxel + 1μmol/L Cisplatinμ 5: Pre-5nmol/L Docetaxel + 1μmol/L Cisplatinμ 6: Pre-1μmol/L Cisplatin + 5nmol/L Docetaxel.

Discussion

The recently demonstrated clinical activity of docetaxel against a variety of malignancies, especially ovarian and breast cancers is encouraging. Clinical trials of the combination of docetaxel and cisplatin have been initiated in ovarian cancer, using empirically derived schedules[6]. Combination therapy with multiple drugs or with multiple modalities is common practice in the treatment of cancers. The purpose of using anticancer drugs in combination is to achieve therapeutic effects greater than those provided by a single drug alone. An optimal protocol of combination chemotherapy may increase the therapeutic efficacy, decrease toxicity toward the host or non-target tissues and minimize or delay the development of drug resistance. Therefore, in vitro investigation of anticancer drugs combination will provide the information or clues to the possible mechanisms of drugs interactions and guidance to clinical implication.

It is aware so far that there are few reports about in vitro interaction between docetaxel and cisplatin. In this study, we investigated the cytotoxic interaction between docetaxel and cisplatin against human breast cancer cells in vitro. Our results demonstrated that docetaxel could be beneficially combination with cisplatin in treatment of the BCap37 cell line. The antineoplastic efficacy was dependent on the sequence in which drug was exposed. The optimal schedule for this combination may be the sequential administration of docetaxel followed by cisplatin.

To analyze the possible mechanism by which cisplatin interacts with the cytotoxic effects of docetaxel, a series of experiments including DNA fragmentation, flow cytometry and western blot were carried out. The results demonstrated that cisplatin affected the cytotoxic effects of docetaxel on both mitotic arrest and apoptosis. Instead, pretreatment with cisplatin could significantly block docetaxel-induced apoptosis. We performed Western blot analysis and determined that docetaxel can induce bcl-2 phosphorylation; interestingly, the combination of docetaxel with cisplatin blocked the bcl-2 phosphorylation. These findings may provide a piece of evidence that cisplatin antagonizes the cytotoxic effects of docetaxel.

In conclusion, this study investigates the possible influence of cisplatin on the effect of docetaxel against human breast cancer cells in vitro. Our results demonstrate the antagonistic interaction occurred when cells were exposed to two drugs simultaneously or exposed to cisplatin before docetaxel. So we suggest that it might be carefully considerated that the optimal schedule for the combination of docetaxel with cisplatin is to adminstrate with docetaxel before cisplatin or the combination of these two drugs.

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收稿日期:2002-04-02

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