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Synergy of 5-aza-2′-deoxycytidine (DAC) and paclitaxel in both androgen-dependent and -independent prostate cancer cell lines

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ABSTRACT

To determine the synergy of 5-aza-2′-deoxycytidine (DAC) and paclitaxel (PTX) against prostate carcinoma (PC) cells by isobolographic analysis. We demonstrated that DAC could significantly increase the susceptibility of PC cells to PTX, and confirmed the synergy of DAC and PTX. DAC enhanced the PTX induced up-regulation of caspase activity and anti-proliferative effect, resulting in an increase of cells in subG1 and G2/M phases. In addition, the synergy was observed in both androgen-dependent and -independent PC cell lines. It suggested that combination chemotherapy with DAC and PTX might be a new strategy to improve the clinical response rate of PC.

1. Introduction

Prostate carcinoma (PC) is the most common malignancy among American males and is the second-leading cause of cancer-related mortality. Although radical prostatectomy and radiation therapy offer hope for a cure for the majority of patients with localized tumors, undetectable residual tumor cells at local or distant sites eventually become clinically evident and lead to recurrence [1]. Most patients that responded initially to ablation of androgen through orchiectomy, luteinizing hormone-releasing hormone agonists and non-steroidal anti-androgens will eventually develop progressive disease [2]. Once androgen-independent PC occurs, prognosis is poor, with a median survival from 7 to 12 months [3,4]. Although chemotherapy has an established role in the treatment of PC and various strategies have been pursued to improve the outcome of PC, more effective combined treatment should be developed to overcome this malignancy [5].

Recently, there has been much emphasis on the critical role of DNA methylation in carcinogenesis, and hypermethylation of cancer-associated genes is prevalent in malignant tumors. 5-aza-2′-deoxycytidine (DAC), a DNA methyltransferase inhibitor, has been used to reverse methylation and reactivate the expression of silenced genes. DAC could suppress the growth of various tumors in vitro, and showed clinical utility against hematopoietic malignancies [6], studies indicated that DAC had clinical activity against metastatic lung carcinoma [7] and is a well tolerated regimen against androgen-independent PC [8]. Several studies have been carried out to examine the synergistic effects of DAC and chemotherapeutic agents against tumor cells, DAC was reported to increase the cytotoxicity of cisplatin (CDDP) and sensitize lung cancer cells to CDDP treatment [9], moreover, a combination of DAC and CDDP showed synergy in triggering apoptotic death of PC cells [10].

In the present study, we demonstrated the synergistic growth suppression of DAC and paclitaxel (PTX) in PC cell lines by isobolographic analysis. The increased susceptibility of PC cells to PTX induced by DAC suggested that combination chemotherapy with PTX and DAC may be a

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new therapeutic option for both androgen-dependent and -independent PC.

2. Materials and methods

2.1. Cell lines and agents

PC cell lines: LNCap (androgen-dependent PC cell line), Du145 and PC3 (androgen-independent PC cell lines) cells were used. AILNCap is an androgen-independent PC cell line that was established as described in reference [11], LNCap cells were maintained in phenol-red-free RPMI supplemented with 10% charcoal-stripped fetal bovine serum, with a change of this steroid-free medium every 3–4 days over 3 months. All PC cell lines were cultured in complete medium and maintained as monolayers and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. DAC and PTX were purchased from Sigma. Caspase 3 inhibitor (DEVD-CHO) and caspase 9 inhibitor (Z-LEHD-FMK) were obtained from Calbiochem (Calbiochem, Darmstadt, Germany).

2.2. WST-1 assay

The effects of agents on cells were determined by the WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) assay. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. Exponentially growing cells were harvested and seeded at 1000 cells/well in a 96-well microtiter plate. After 4 h incubation, agents or penicillin/streptomycin-free medium as the untreated control were added, followed by continuous incubation for 72 h. Ten microliters of WST-1 (Roche, Penzberg, Germany) were added to each well and the incubation was continued for 2 h. The absorbance was measured with a microculture plate reader at 450 nm. The percent of cytotoxicity was calculated using the following formula: % Cytotoxicity = [1 – (absorbance of experimental well-absorbance of blank)/(absorbance of untreated control well-absorbance of blank)] × 100%.

2.3. Flow cytometric analysis

Apoptosis induction was examined by flowcytometric analysis, cell lines were cultured with DAC alone or chemotherapeutic agent in the absence or presence of DAC for 72 h, and then cells were collected and fixed with 70% ethanol at −20 °C overnight, washed three times with PBS and incubated for 30 min in 7-Amino-Actinomycin D (7-AAD) staining solution (BD Biosciences, San Jose, USA). 7-AAD is a convenient nucleic acid dye that can be used in flow cytometric assays. Cell counts in each phase of the cell cycle were estimated using a FACSCalibur (BD Biosciences, San Jose, USA) and Cellquest 3.0 software.

2.4. Detection of caspase activity

The activities of caspase 3 and 9 were measured using an APOPCYTO Caspase Colorimetric Assay Kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan). Protein was extracted using ice-cold cell lysis buffer and two hundred micrograms of total protein in 50 μl of lysis buffer, 50 μl of 2 × reaction buffer containing 10 mM DTT and 5 μl of caspase 3 or 9 substrate was added into each well of a 96-well microplate, and after incubation at 37 °C for 16 h, the absorbance representing the formation of p-nitroanilide was measured with a microculture plate reader at 405 nm.

2.5. Western blotting

PCNA expression was analyzed by Western blotting, protein was extracted using ice-cold cell lysis buffer and heat 30 μg sample to 95 °C for 5 min, cool on ice. SDS polyacrylamide gel electrophoresis was performed and electrotransfer to PVDF membrane. PCNA monoclonal antibody (PC10, antibody dilution 1:2000) was purchased from Sigma and anti β-actin monoclonal antibody (Abcam, Cambridge, UK) was used as an internal control. Quantitative PCNA expression levels were measured using BandScan software.

2.6. Statistical analysis

All determinations were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). Statistical significance was determined by Student's *t* test, and *P* value of 0.05 or less was considered significant. Synergy was evaluated by isobolographic analysis as described by Berenbaum [12]. Fractional inhibitory concentration = the dosage of IC_{50} by each agent in combination/the dosage of IC_{50} by each agent alone, whether the combination is additive, synergistic or antagonistic is shown by whether the point lies on, below or above the straight line joining the dosages of the two drugs that, when given alone, produce the same effect as that of the combination in isobolographic analysis.

3. Results

3.1. Synergistic growth suppression by DAC and PTX

DAC (Fig. 1A) and PTX (Fig. 1B and C, results for LNCap and AILNCap are shown) each caused dosage-dependent cell growth suppression of PC cells. DAC could increase the susceptibility of PC cells to PTX (Fig. 1B and C, results for LNCap and AILNCap are shown), and combined treatment with DAC and PTX caused synergistic growth suppression in all PC cell lines examined, as shown by isobolographic analysis (Fig. 1D).

3.2. Induction of apoptosis and cell cycle arrest by DAC and/or PTX

To clarify the mechanism of the synergy of DAC and PTX, we analyzed the induction of apoptosis by DAC alone or combined with PTX in four PC cell lines. Flow cytometric analysis revealed that DAC did not significantly induce apoptosis at any concentration, rather, it caused cell cycle arrest at G2/M (Fig. 2A, results for 0.1 and 1 μM in LNCap are shown). On the other hand, PTX induced both apoptosis and cell cycle arrest at G2/M in a dosage-dependent manner. Furthermore, at 0.1 and 1 μM, DAC could promote the induction of both apoptosis and G2/M arrest by PTX in these cell lines (Fig. 2B–D, results for 0.5 nM PTX in LNCap and AILNCap, 2.5 nM in PC3 and Du145 are shown).

3.3. Detection of caspase activity

Caspase activity was detected in four PC cell lines treated with DAC alone or DAC combined with PTX. DAC did not affect the activity of cas-