

## Original Article

# Optimal concentration of carboplatin combined methotrexate in suppressing prostate cancer PC-3 cell and related mechanism

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**Abstract:** Prostate cancer is the most common male reproductive tract malignant tumor with high incidences. Traditional chemotherapy drugs killed tumor by inducing cancer cells apoptosis. However, the therapeutic effect of single medication has reached plateau. We adopted drug combination to investigate the best chemotherapy concentration of carboplatin combined with methotrexate in suppressing prostate cancer PC-3 cell and related mechanism. PC-3 cells were treated with different concentrations combined drugs (carboplatin, 20, 30, 40, 50, 60  $\mu\text{g}/\text{ml}$ ; methotrexate, 0.20, 0.30  $\mu\text{g}/\text{ml}$ ). MTT was applied to test cell apoptosis. Hematoxylin and acridine orange (AO)/ethidium bromide (EB) staining together with immunohistochemical experiment were used to investigate the effect of drug combination on cell morphology. The ideal concentration of carboplatin and methotrexate in combination was 40-50  $\mu\text{g}/\text{ml}$  and 0.20  $\mu\text{g}/\text{ml}$ , respectively. Carboplatin concentration at 60  $\mu\text{g}/\text{ml}$  showed the highest inhibition rate. Cell staining, morphological observation, and immunohistochemical results revealed that drug combination at abovementioned concentration achieved the goal of control tumor cells by inducing cell apoptosis. The best concentration was carboplatin at 60  $\mu\text{g}/\text{ml}$  and methotrexate at 0.20  $\mu\text{g}/\text{ml}$  under drug combination. Considering the possible toxicity of carboplatin, we suggest the optimal concentration should be carboplatin at 40  $\mu\text{g}/\text{ml}$  and methotrexate at 0.20  $\mu\text{g}/\text{ml}$ .

**Keywords:** Carboplatin, methotrexate, drug combination, prostate cancer, chemotherapy, cell apoptosis

## Introduction

Prostate cancer is a common malignant tumor only second to liver cancer in Asian men. It has the leading incidence in cancer worldwide and is the most common male reproductive tract malignant tumor. In our country, in spite of its low incidence, prostate cancer still reached 6.41/100,000 in male [1-5]. Furthermore, its incidence presents obvious increasing trend following aging of population and living condition improvement. For example, its incidence in Shanghai elevated from 2.9/100,000 in 1991 to 8/100,000 in 2013 [1-5]. In recent years, inducing malignant tumor apoptosis has become a new hot spot for malignant tumor treatment. Apoptosis is positively and negatively regulated by a series of genes. Cancer often appears gene mutations, deletion, abnormal expression, or dysfunction [6, 7]. For instance, tumor suppressor gene P53 and Bcl-2 family

can synergistically induce cell apoptosis by shifting cells from G phase to S phase to eliminate abnormal cells [8, 9].

Carboplatin came into the market first in the UK in 1986. As a second generation platinum compound, it has similar biochemical character to cisplatin. Its mechanism is similar to alkylating agent with cell cycle non-specificity [10]. Carboplatin can kill the cells by inter- or intra-strand cross-linking with nuclear DNA. It can cause DNA damage, resulting in DNA replication and transcription injury. Therefore, it can block the cells in G2 phase to prevent further cell division [10, 11]. On pharmacokinetic aspects, carboplatin shows similar distribution with cisplatin that mainly in the liver, kidney, skin and tumor. However, its plasma protein binding rate is very low and irreversible. Its half-life  $\gamma$  phase is at least five days,  $\alpha$  phase is 1~2 h, and  $\beta$  phase is 2.6~5.9 h. Carboplatin is mainly excreted by

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**Table 1.** Drug concentration gradient design

Group	Methotrexate ( $\mu\text{g/ml}$ )	Carboplatin ( $\mu\text{g/ml}$ )
1	0.20	20.0
2	0.20	30.0
3	0.20	40.0
4	0.20	50.0
5	0.20	60.0
6	0.30	20.0
7	0.30	30.0
8	0.30	40.0
9	0.30	50.0
10	0.30	60.0

kidney. When the creatinine clearance reaches 60 ml/min, it is removed by kidney to 71% within 24 h. 65% of the dosage is discharged in the first 12 h, 6% in the next 12 h, only 3%~5% is removed after 96 h. It is unclear whether the rest of the drug is eliminated through bile or other ways [12]. Active platinum can be found in 24 h urine, suggesting that only tiny drug metabolized in the body. Patients with low creatinine clearance rate show longer half-life, thus the dose should be adjusted. Carboplatin is not secreted by renal tubule, which may be the reason of its lower renal toxicity than cisplatin. It has remarkable curative effect to small cell lung cancer, ovarian cancer, testicular cancer, and head and neck squamous cell carcinoma [13-15].

As a traditional antitumor drug, methotrexate is also widely used in clinic. It has high affinity with dihydrofolate reductase. It can block enzyme activity by competitive binding, leading to folic acid cannot transform to tetrahydrofolic acid. The later prevent deoxyuridine acid transform to DNA thymine nucleotide, thus blocking DNA de novo synthesis pathway [16]. Methotrexate, meanwhile, also can block purine nucleotide biosynthesis, thus interfering RNA and proteins synthesis, inhibiting cell chromosome replication to prevent tumor cell DNA synthesis, inhibit tumor cell growth, division, and proliferation [17, 18].

Research showed that at 48 h after single medication, the optimum concentration of carboplatin is 40  $\mu\text{g/ml}$ , while the effective concentration of methotrexate is 0.05~0.5  $\mu\text{g/ml}$  [14, 17]. But the effect of two drugs combination on prostate cancer is still unclear.

This study used different doses of carboplatin and methotrexate on prostate cancer PC-3 cells for 48 h, and detected its effect through cell staining, morphological observation, immunohistochemistry, and MTT to explore the optimal combination concentration. We aimed to clarify the effect and molecular mechanism of the two drugs in treating prostate cancer.

### Materials and methods

#### Materials

Carboplatin and methotrexate were purchased from Guangzhou Paekche Antitumor Drug Store. Prostate cancer PC-3 cells were bought from Wuhan University experiment animal center. Normal saline, 95% ethanol, and PBS were purchased from Guangzhou chemical plant. Hematoxylin dyeing liquid, 0.01% acridine orange (AO), 0.01% ethidium bromide (EB), and MTT were provided by Takara. P53 fluorescent antibody was from P&D. Instruments used include: fluorescent microscope (Nikon, Japan), inverted microscope (Nikon, Japan), enzyme-linked immune detector (Thermo, USA), tweezers, petri dishes, non-fluorescence slide, cover glass, blotting paper, etc.

#### Drug concentration design

We designed different concentrations of carboplatin and methotrexate. Among them, the carboplatin concentration was divided into 20, 30, 40, 50, and 60  $\mu\text{g/ml}$ , while methotrexate was divided into 0.20 and 0.30  $\mu\text{g/ml}$ . A total of 12 groups including two blank controls were determined by MTT (**Table 1**). Morphological observation and immunohistochemical detection were applied, including control group, methotrexate 0  $\mu\text{g/ml}$  and carboplatin 0  $\mu\text{g/ml}$ ; experimental group 1, methotrexate 0.2  $\mu\text{g/ml}$  and carboplatin 40  $\mu\text{g/ml}$ ; experiment group 2, methotrexate 0.3  $\mu\text{g/ml}$  and carboplatin 40  $\mu\text{g/ml}$ .

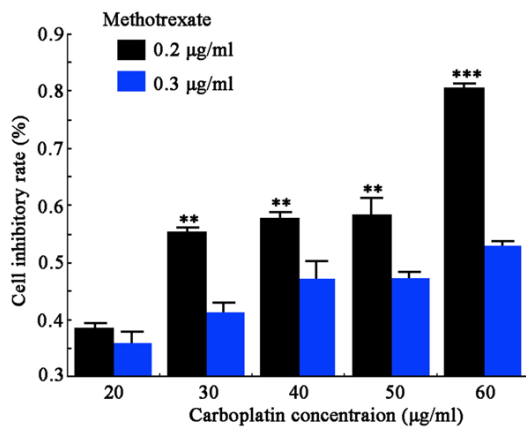
#### Cell culture

Under aseptic condition, three dry cover glasses were put in a petri dish. PC-3 cells in logarithmic phase were digested with 0.25% trypsin to prepare cell suspension. The cells were seeded in petri dish and cultured for 24 h. Then the cells were treated with 0.2  $\mu\text{g/ml}$  methotrexate and 60  $\mu\text{g/ml}$  carboplatin for 48 h to

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**Table 2.** MTT assay detection of cell proliferation inhibition (n = 30)

Group	Methotrexate (µg/ml)	Carboplatin (µg/ml)	Mean OD value	Relative OD value	Cell proliferation inhibitory rate
Zero	0	0	0.079	-	-
Control	0	0	0.231	0.152	-
1	0.2	20	0.158	0.079	0.3811±0.011
2	0.2	30	0.132	0.053	0.5519±0.010
3	0.2	40	0.129	0.050	0.5749±0.015
4	0.2	50	0.128	0.049	0.5814±0.032
5	0.2	60	0.103	0.024	0.8040±0.009
6	0.3	20	0.146	0.067	0.3583±0.022
7	0.3	30	0.138	0.059	0.4108±0.016
8	0.3	40	0.129	0.050	0.4700±0.034
9	0.3	50	0.129	0.050	0.4716±0.010
10	0.3	60	0.113	0.034	0.5267±0.011



**Figure 1.** MTT assay detection of sample OD value. N = 30. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

prepare growth slide. The cell concentration in suspension was adjusted to  $2\sim 4 \times 10^4$ /ml. 4000~8000 cells were seeded in 96-well plate for 24 h and treated by different concentration of drug combination. Each concentration was repeated for 30 times.

### MTT assay

The cells cultured for 3 days in 96-well plate were observed on inverted microscope. 20 µl 5 mg/ml MTT solution was added to each well for 4 h, and then 150 µl DMSO was added for 30 min after removing the supernatant. The plate was read on microplate reader at 490 nm to obtain the optical density (OD) value.

In this study, we designed different concentration combination and set the zero group (medi-

um, MTT, DMSO) and control group (cell, medium, MTT, DMSO). PC-3 cells were tested for OD value after 48 h treatment. Relative OD value was obtained after OD value in each group minus that in zero group.

Cell proliferation inhibitory rate = (OD value in control-OD value in test)/OD value in control [formula 1-1].

### Hematoxylin staining

After washed by normal saline, the slide was fixed by 95% ethanol for 5 min. After further washed by PBS for two times, the slide was stained with hematoxylin for 5 min and observed under light microscope. Each group was repeated for five times.

### AO/EB staining

After washed by normal saline, the slide was fixed by 95% ethanol for 5 min. After further washed by PBS for two times, the slide was stained with 0.01% AO + 0.01% EB for 5 min and observed under light microscope. Each group was repeated for five times.

### Immunohistochemistry

The slide was fixed by cold acetone for 5 min, and then treated by normal goat serum at 37°C for 30 min. P53 primary antibody and specific fluorescent secondary antibody were successively instilled to the slide at 37°C for 2 h and 1 h, respectively. After sealed by glycerin, the slide was observed under light microscope. Each group was repeated for five times.

### Statistical analysis

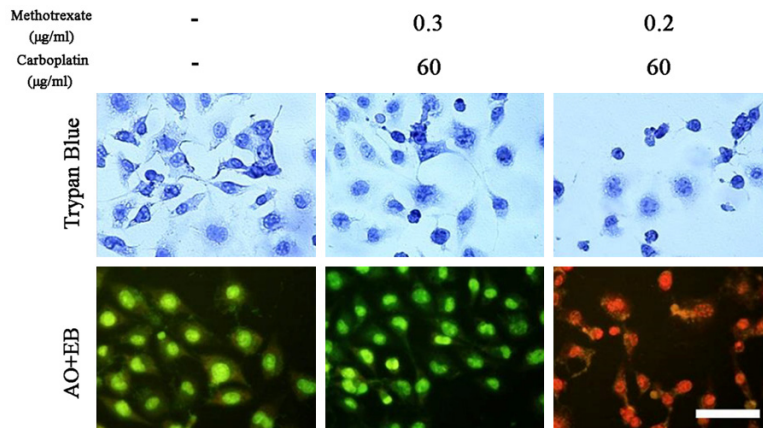
The data was treated by Origin 8.0 software and analyzed by SPSS13.0 software.

## Results

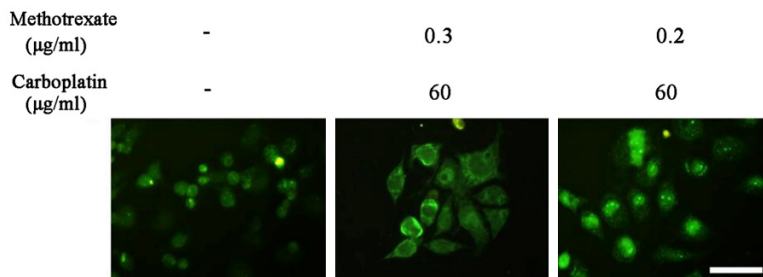
### MTT detection of OD value

To preliminary explore the effect of combination therapy, we designed different concentra-

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**Figure 2.** Hematoxylin and AO/EB staining detection of cell apoptosis. All experiments were repeated for five times. Measuring scale = 80  $\mu\text{m}$ .



**Figure 3.** Immunohistochemistry detection of p53 expression. All experiments were repeated for five times. Measuring scale = 40  $\mu\text{m}$ .

tions of drug combinations (**Table 1**) and tested OD value by MTT method (**Table 2**). We used [formula 1-1] to show proliferation inhibitory rate. According to the formula, positive result means inhibitory effect on cells; zero means no effect, and negative result means promoting effect.

As shown in **Figure 1**, drug's inhibitory effect on PC-3 cells enhanced following carboplatin concentration increasing. Inhibition rate curve presented S type. Inhibition rate curve appeared plateau when carboplatin at 40~50  $\mu\text{g/ml}$ . Methotrexate also has an effect on experimental result. MTT assay revealed that under drug combination, methotrexate at 0.2  $\mu\text{g/ml}$  had more obvious inhibitory effect on PC-3 cells when the carboplatin concentration greater than 30  $\mu\text{g/ml}$ . However, methotrexate concentration changes had significant impact on cell proliferation when carboplatin concentration at the plateau of 40-50  $\mu\text{g/ml}$ . The best combination inhibitory effect appeared in the methotrexate at 0.2  $\mu\text{g/ml}$  and carboplatin at 60  $\mu\text{g/ml}$ .

### Cell staining and morphological observation

To test the best inhibitory effect of drug combination, we treated the cells with 0.2 or 0.3  $\mu\text{g/ml}$  methotrexate and 60  $\mu\text{g/ml}$  carboplatin for 48 h to induce cell apoptosis. Then we applied hematoxylin and AO/EB staining method to observe cell morphology.

As shown in **Figure 2**, PC-3 cells appeared classical apoptotic status under two drugs combination: nucleus pycnosis, fracture, irregular edge, higher density of chromatin, and nucleolus cracking. Some cells even presented late apoptosis: membrane buckling, crimping, foaming, and form apoptotic body. However, 0.2  $\mu\text{g/ml}$  methotrexate and 60  $\mu\text{g/ml}$  carboplatin showed more obvious apoptotic effect.

Similarly, AO/EB staining showed that normal PC-3 cells presented green nucleus and dark red cytoplasm. 0.3  $\mu\text{g/ml}$  methotrexate and 60  $\mu\text{g/ml}$  carboplatin treatment made bright green spots in nucleus, yellow green nuclear chromatin concentration, and nucleus began cracking into pieces. In the third group, 0.2  $\mu\text{g/ml}$  methotrexate and 60  $\mu\text{g/ml}$  carboplatin treatment let PC-3 cells present obvious titian, nuclei deformation and breakage, and chromosome aggregation.

### Immunohistochemical result

To further investigate the related mechanism of drug combination suppressing cell proliferation, we performed immunohistochemistry to detect p53 expression.

As shown in **Figure 3**, PC-3 cells in two experiment groups showed green nucleus. It indicated that p53 overexpression in PC-3 cells and mainly located in nucleus.

### Discussion

As one of common malignant tumors, prostate cancer has reached the bottleneck in chemo-

therapy. New targeted drugs and drug combination to inhibit tumor growth and even induce cell apoptosis is the main direction of the present study [4, 5].

Methotrexate is a type of antifolates antitumor drug with high affinity to dihydrofolate reductase. It can block enzyme activity through competitive inhibition, leading to folic acid cannot transform to tetrahydrofolic acid to play a role of coenzyme [19]. Clinical trial confirmed that the effective concentration of methotrexate in inhibiting tumor growth was 0.05-0.5  $\mu\text{g/ml}$ . Deoxyuridine acid cannot be transformed into DNA thymine nucleotide under such concentration. At the same time, it also prevent purine nucleotide biosynthesis to some extent, leading to DNA de novo synthesis pathway blockage, RNA and protein synthesis interference, and chromosome replication inhibition to hinder tumor cell growth and inhibit tumor cell proliferation [20]. Our results also presented that methotrexate inhibitory effect on PC-3 cell proliferation was not completely positive correlated with concentration. Low dose methotrexate had better suppression effect on cancer cell proliferation.

Carboplatin has high curative effect on most common malignant tumors with less kidney and gastrointestinal tract toxicity. It has been treated as a common drug used for multiple tumor chemotherapy. It can intra- or inter-strand cross-linking with DNA, leading to DNA replication and transcription damage, thus retard cell division in G2 phase [21]. The optimal concentration of carboplatin is 40  $\mu\text{g/ml}$  when single medication. Lower concentration has no obvious effect on cancer cells, while higher concentration may impact normal tissue cells [22]. This experiment also demonstrated that carboplatin inhibition on PC-3 cell proliferation reached plateau at 40~50  $\mu\text{g/ml}$ , whereas carboplatin at 60  $\mu\text{g/ml}$  showed most significant inhibition effect.

Meanwhile, MTT results also revealed that methotrexate concentration changes did not have markedly difference on PC-3 cell proliferation inhibition in the plateau. It suggested that we should use lower methotrexate to reduce the toxicity in drug combination with carboplatin at 40~50  $\mu\text{g/ml}$ . Similarly, carboplatin  $\geq$  30  $\mu\text{g/ml}$  showed more obvious inhibition effect with 0.2  $\mu\text{g/ml}$  methotrexate. Compared with

0.3  $\mu\text{g/ml}$ , methotrexate at 0.2  $\mu\text{g/ml}$  presented smoother curve and more stable inhibition effect with carboplatin at 30-50  $\mu\text{g/ml}$ . It indicated that combination of 40  $\mu\text{g/ml}$  carboplatin and 0.2  $\mu\text{g/ml}$  methotrexate provided larger carboplatin concentration range. Thus, we could restrain side effect, even reduce toxic reaction by controlling carboplatin dose.

Cell staining and morphology observation demonstrated that a large amount of PC-3 cells appeared typical apoptosis when carboplatin was 60  $\mu\text{g/ml}$  and methotrexate was 0.2  $\mu\text{g/ml}$ . Immunohistochemical results also showed that p53 overexpressed in the cells, suggesting that carboplatin mainly reduce cell number by inducing apoptosis under such concentration. In other words, large dose of carboplatin not only inhibits tumor cells proliferation, even causes cell apoptosis. Though its mechanism is still unclear, carboplatin at such concentration may also cause normal tissue cell apoptosis, thus produce side effect.

The ideal drug concentration under drug combinations is carboplatin at 40-50  $\mu\text{g/ml}$  and methotrexate at 0.2  $\mu\text{g/ml}$ . We can reduce the side effect by controlling the dosage ratio. Carboplatin concentration higher than 60  $\mu\text{g/ml}$  may induce tumor cell apoptosis, which may be the main cause of toxic effects.

### Disclosure of conflict of interest

None.

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