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Original Research Article

In vitro evaluation of combination effects of doxorubicin with methylxanthine fractions isolated from Bancha and Pu-erh teas against breast cancer cells

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ABSTRACT

Background: In the present study we investigated the combination effects of anthracycline antibiotic, doxorubicin, with methylxanthine fractions isolated from Bancha and Pu-erh tea leaves, against MCF-7 and MDA-MB-231 breast cancer cell lines.
Methods: Neutral red uptake assay was used for assessment of cytotoxicity effects and fractional effect analysis and combination index for evaluation of the combination effects.
Results: Doxorubicin was used in varying concentrations by a double dilution method, whereas the methylxanthine fractions were in fixed concentrations – 100, 200, 400 or 600 µg/ml. Results have shown that methylxanthine fraction isolated from Bancha has synergic effects with doxorubicin, while methylxanthines from Pu-erh displayed antagonistic effects.
Conclusions: The obtained results lead us to suspect, that even minor differences in the composition of natural products can lead to significant differences in the biological activity of the product.

Keywords: Pu-erh, Bancha, Methylxanthine, Breast cancer, Combination effect, Synergism

INTRODUCTION

Methylxanthines, caffeine, theophylline and theobromine are found in the most commonly consumed beverages and foods worldwide. The main effects observed after consumption of methylxanthines include excitation of the central nervous system, strengthening of the heart activity and pulse increase, improvement of organ perfusion, bronchodilation, stimulation of urination and intestinal peristalsis.[1] Although there are many mechanisms of action described, most pharmacological effects of methylxanthines result from a blockade of the adenosine receptors.[2] The both used teas in our study - Pu-erh and Bancha, contain significant amounts of methylxanthines.[3]

Anthracycline antibiotic, doxorubicin, is widely used antineoplastic drug in the therapy of variety of human solid and hematological malignancies.[4] Doxorubicin alone or in combinations is the mainstay therapy of the metastatic breast cancer. However, dose-limiting

toxicities such as cardiotoxicity and bone marrow toxicity limit its widespread use.

To increase the therapeutic activity on one hand, and to reduce the adverse drug effects on the other hand, in the oncological practice, it is often recoursed the combination chemotherapy regimens.[5] Therefore, the purpose of the present study is to evaluate combination effects of doxorubicin with methylxanthines isolated from Pu-erh (MXP) and Bancha (MXB) teas against two breast cancer cell lines - MCF-7 and MDA-MB-231.

METHODS

Plant material

The air dried Pu-erh and Bancha tea leaves were purchased from the local market and the material was held at room temperature. They have been identified by Assoc. Prof. Iliya Slavov from the Department of Biology, Faculty of Pharmacy, Medical University of Varna, Bulgaria. Before extraction the obtained plant material was ground into small particles.

Extraction of methylxanthines

Accurately weighed amounts of Pu-erh and Bancha tea leaves (50 g) were extracted under reflux with distilled water for 60 min and filtered through a Buchner funnel. The aqueous extracts were acidified with sulfuric acid and concentrated. The solution was extracted with chloroform in separating funnel. Chloroform extract was washed with sodium hydroxide solution and then with water. After evaporation of chloroform, a mixture of methylxanthines was obtained and its percentage yield was calculated.[6]

Cell cultures

The breast cancer cell lines MCF-7 (estrogen, progesterone receptors +, HER2-), and MDA-MB-231 (triple negative, ER-, PR-,HER-) cells were cultured in Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco, Austria), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Lonza, Belgium) under 5% CO_2 and 95% air atmosphere at 37^{o}C. Plastic flasks 25 cm^2 supplied by Greiner, Germany, were used to grow the cells. The cells were kept in exponential phase of growth and after processing with trypsin-EDTA (FlowLab, Australia) they were seeded into 96-well plates (100 µl/well) at a density 1×10^4 cells/ml.

Cell viability assay

Cell viability was evaluated in MCF-7 and MDA-MB-231 cells by neutral red uptake assay according to the standard protocol, with some modifications, which is based on the accumulation of the neutral red dye in lysosomes of viable cells.[7] MCF-7 and MDA-MB-231 cells were seeded into 96-well plates. The cells were incubated with the used compounds for 72 h, and cell viability was determined later. The treatment medium was replaced with DMEM containing 100 µg/mL neutral red dye and the cells were incubated for three hours. The optical density of the samples was measured by a microplate reader (TECAN, Sunrise TM, Groedig/ Salzburg, Austria) at 540 nm. Cell viability was expressed as percent of control values.

Analysis of drug combinations

To evaluate the combination effects, we used fractional effect analysis (FA) or Bliss independence.[8,9] The effect in this method is considered synergistic when the observed effect is greater than the effects of each individual agent. The equation used for calculating the combining effect (CE) is:

$$CE = \frac{A_1 \times B_2}{100}$$

Where A_1 and B_2 are inhibition percentages with single agents, expressed as percentage of the untreated control. For each concentration, we calculated the theoretical values, which we further compared with those we received actually: for $CE_{measured} = CE_{calculated}$ we conceded the effect as additive; for $CE_{measured} < CE_{calculated}$ as synergistic; and for $CE_{measured} > CE_{calculated}$ as antagonistic.

The second method we used was Chou-Talalay method.[10] We recourse to this method, when we have observed significant synergistic effects, to confirm them. In our study, the CI values were determined for combination of doxorubicin and MXB using CalcuSyn® software (Biosoft, Cambridge, UK).[11] A CI value less than 0.9 indicates synergism, CI equal to 0.9-1.10 indicates additive interaction, and CI greater than 1.10 indicates antagonism.

Statistical analysis

The statistical evaluation was performed using GraphPad Prism 8.01 software (GraphPad Software, USA), and the significant differences between groups were analyzed using ANOVA. All results are expressed as arithmetic means±standard deviation (SD) of three separate experiments (each experiment was done with three parallels). A difference at $p<0.05$ was considered statistically significant.

RESULTS

In our previous work, we defined the sensitivity of the used cell lines to doxorubicin. The both cell lines showed dose-dependent decreases in cell viability compared to the negative control (untreated cells). The IC_{50} value against MCF-7 cells were 0.07 µM, while against MDA-MB-231 were 0.79 µM (Table 1).

Table 1: Doxorubicin IC_{50} values with 95% CI against MCF-7 and MDA-MB-231 cell lines.

	MCF-7	MDA-MB-231
Doxorubicin IC_{50} (95% CI)	0.07 µM (0.02173 to 0.1160)	0.79 µM (0.25353-1,0654)

As can be seen on the Table 1, MCF-7 cells are approximately ten times more sensitive to the action of doxorubicin.

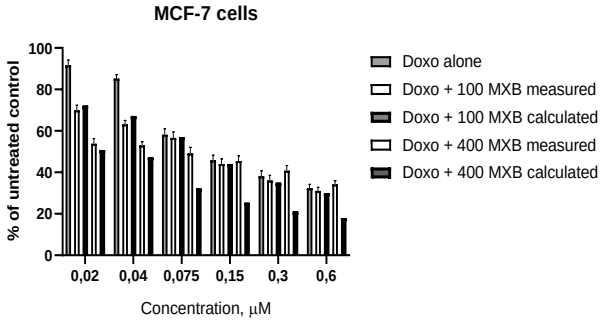

Figure 1: MCF-7 cells treated with Doxorubicin (Doxo) alone in concentration range 0.02-0.6 µM and in combination with 100 and 400 µg/ml methylxanthines isolated from Bancha (MXB).

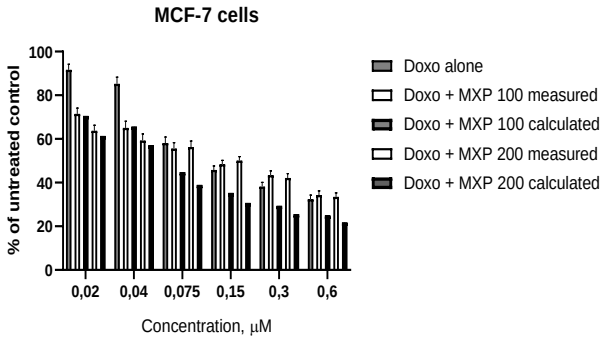

Figure 2: MCF-7 cells treated with Doxorubicin (Doxo) alone in concentration range 0.02-0.6 µM and in combination with 100 and 200 µg/ml methylxanthines isolated from Pu-erh (MXP).

To determine whether methylxanthine isolated from Bancha (MXB) and Pu-erh (MXP) could sensitizes MCF-7 cells to treatment with doxorubicin, we cultured cells with doxorubicin at concentration range of 0.02 to 0.6 µM, alone or in combination with fixed 100 and 400 µg/ml MXB; 100 and 200 µg/ml MXP, for 72 hours. The cell proliferation was assessed using neutral red uptake assay. The results from treatment of MCF-7 cells are shown in Figure 1 and 2.

As can be seen from Figure 1, lower concentrations of doxorubicin (0.02-0.04 µM) with 100 µg/ml MXB have synergistic effects and with an increase in the concentrations of doxorubicin (0.075-0.6 µM) the effects are additive. The usage of higher concentration of MXB - 400 µg/ml, leads to antagonistic effects. The situation with MXP was similar. We observe additive effects in lower concentrations of doxorubicin (0.02-0.04 µM) with both used concentrations of MXP. With the other used concentrations the effects were antagonistic.

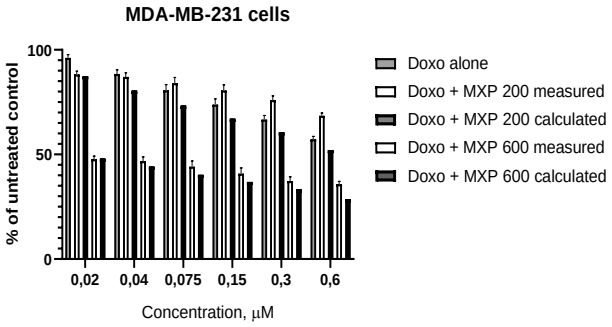

Figure 3: MDA-MB-231 cell line treated with Doxorubicin (Doxo) alone in concentration range 0.02-0.6 and in combination with 200 and 600 µg/ml methylxanthines isolated from Pu-erh (MXP).

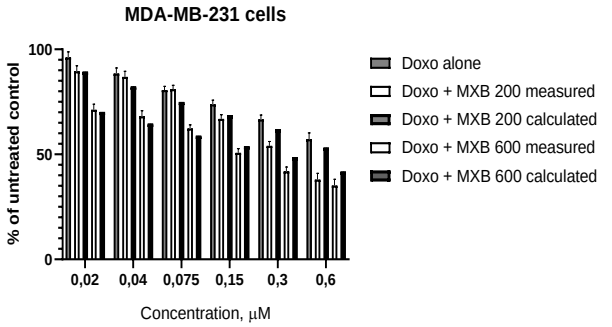

Figure 4: MDA-MB-231 cell line treated with Doxorubicin (Doxo) alone in concentration range 0.02-0.6 µM and in combination with 200 and 600 µg/ml methylxanthines isolated from Bancha (MXB).

We tested combinations effects of doxorubicin and MXB and MXP on MDA-MB-231 cell line. Because MDA-MB-231 cell line was more resistant to the action of doxorubicin, we used higher concentrations of methylxanthines - 200 and 600 µg/ml. The results are presented on Figure 3 and 4.

The MXP only at the lowest concentration of doxorubicin (0.02 µM) provided additive effects, all other concentrations showed antagonistic effects (Figure 3).

The situation with MXB was completely different. In the lower concentrations, doxorubicin (0.02-0.075 µM) and MXB (200 and 600 µg/ml) have additive effects, while in the highest concentrations (0.15-0.6 µM) the combination is with significantly expressed synergistic effect (Figure 4).

Table 2: Combination index after treatment of MDA-MB-231 cells with combination doxorubicin (0.02-0.6 µM) + MXB (200 and 600 µg/ml).

Doxorubicin concentration (µM)	MXB concentration (µg/ml)	CI	MXB concentration (µg/ml)	CI
0.02	200.0	1.20636	600.0	1.06166
0.04	200.0	1.24558	600.0	1.03197
0.075	200.0	1.21176	600.0	0.93512
0.15	200.0	0.81526	600.0	0.73723
0.3	200.0	0.76702	600.0	0.68348
0.6	200.0	0.59957	600.0	0.75423

CI: Combination index.

To confirm the observed synergistic effects, we used CalcuSyn® software, to calculate the combination index. The results are presented in Table 2.

The calculated combination index indicates that the combination of doxorubicin (0.15-0.6 µM) and MXB (200 and 600 µg/ml) allows synergistic effects.

DISCUSSION

The use of total extracts, fractions or pure substances of plant origin alone or in combination with approved drugs in the treatment of diseases is not something new and is already established in the pharmaceutical researches. Searching for such synergistic plant/herb-drug interactions, which could assist the therapeutic benefit or ameliorate the degree of adverse drug effects, is of particular importance for a field like oncology.[12] Mehylxanthines, and especially the main component caffeine, are one of the most consumed natural products worldwide and they have been the subject of scientific researches frequently.[13]

Considering that the used methylxanthine fractions are rich in caffeine (the amount of caffeine in both fractions was determined by HPLC; results not published yet), we performed a literature review regarding the combination effect of caffeine with conventional antitumor drugs on tumor cells. The literature data have shown that there are various mechanisms, discussed as possible about the action of caffeine against tumor cells and their combination with other chemotherapeutic drugs. The most debated issues are the inhibition of DNA repair and the inhibition of efflux pumps.[14-17] The both mechanisms suggest sensitizing the tumor cells to the action of cytotoxic drugs. On the other hand, there are data, that mehylxanthines ameliorate the pharmacological effects of aromatic anticancer compounds, such as doxorubicin, mitoxantron, topotecan, camptothecin, as well as other mutagenic agents.[18-22] The possible mechanisms proposed by these authors include reducing the possibility of intercalation in the DNA strand and the direct formation of complexes of methylxanthines with aromatic compounds.

In our previously study, we demonstrated weakly expressed antiproliferative effects of MXP.[6] We didn't find synergistic effects on MDA-MB-231 and HT-29 cell lines, when they were combined with oxaliplatin. Similar combination results are reported by other authors. Martins et al demonstrated synthesis of new derivative of caffeine, 6-selenocaffeine, which decreases the cytotoxic effects of oxaliplatin on MCF-7 cells, while increasing the activity of doxorubicin.[23] Hertz et al showed antitumor activity of guarana (*Paullinia cupana*), which is rich in caffeine and catechins, on MCF-7 cells and increased antiproliferative effects when combining with conventional antitumor drugs, such as doxorubicin.[24] The effect of the combinations on cell viability after 24 hours is significantly increased, while after 72 hours it transforms to synergetic antiproliferative effect. The authors suggest that other components, such as catechins, are responsible for these temporal changes. In the present study, the combination of MXP with doxorubicin did not provide synergistic effects. Hill et al explained that with possible interceptor role of caffeine, protecting cancer cell DNA from intercalation.[25] Since the both methylxanthine fractions are similar in composition (as was mentioned above; results not published yet), we did not expect any different effects from MXB. However, the results we received invalidated our presumption. MXB, have shown a notable synergistic effect with doxorubicin on MDA-MB-231 cell line, evaluated by the both used combination methods.

We have suggested, that even minor differences in the composition of a natural product, such as in our case, can lead to significant differences in the biological activity of the product, which requires follow-up studies to confirm this activity.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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