

# Effects of Docetaxel, Doxorubicin and Cyclophosphamide on Human Breast Cancer Cell Line MCF-7

MARIANNA TREBUNOVA<sup>1</sup>, GALINA LAPUTKOVA<sup>1</sup>, EVA SLABA<sup>2</sup>,  
KAMILA LACJAKOVA<sup>3</sup> and ANETA VEREBOVA<sup>1</sup>

*Departments of <sup>1</sup>Medical and Clinical Biophysics and <sup>2</sup>Medical Biology,  
<sup>3</sup>Third Department of Internal Medicine, Faculty of Medicine, P.J. Safarik University, Kosice, Slovakia*

**Abstract.** *Background:* Cell viability assays are important tools in oncological research. The purpose of this study was to evaluate the effect of docetaxel, doxorubicin and cyclophosphamide on the metabolic activity of MCF-7 breast cancer cell line. *Materials and Methods:* Antiproliferative effect of cytostatics on MCF-7 cells was measured using the standard colorimetric test. The MCF-7 cell line was exposed to cytostatics for 24 hours. The metabolic activity was evaluated over the 24 hours. *Results:* According to the statistical analysis, the change in the growth rate was significant ( $p<0.05$ ) for the 120 nM docetaxel and above the 200 nM doxorubicin treatment in comparison with sensitive MCF-7 cells. When considering drugs in combination (60 nM docetaxel with 200 nM doxorubicin and 60 nM docetaxel with 500 nM doxorubicin) after the addition of 600 nM cyclophosphamide, we found a statistically significant decrease of metabolic activity of the MCF-7 cell line ( $p<0.05$ ). *Conclusion:* Cyclophosphamide at 600 nM seems to enhance the influence of docetaxel when combined with doxorubicin.

Cytostatics are most commonly administered as combination therapy. During combination therapy, two different cytostatics with additive or synergistic effects are prescribed at the same time in relatively small doses compared with their use in separate applications. In general, cytostatics selected for combination chemotherapy are active against the tumour when used alone, with different mechanisms of action and minimally overlapping toxicities. Thereby, occurrence of side-effects is considerably reduced, with increased effectiveness

of the therapy. Combination therapy also reduces the risk of resistance to cytostatics, that can develop with the prolonged use of a particular drug (1).

Taxane drugs, paclitaxel and the semisynthetic derivative docetaxel, have emerged as critically important antimicrotubule drugs in the treatment of patients with breast cancer. Taxane treatment causes the stabilization of microtubules against depolymerisation leading to the formation of abnormal bundles of microtubules. Abnormal bundles resist physiological disassembly, accumulate within tumour cells and inhibit cell proliferation, leading to cell cycle arrest at the G<sub>2</sub>/M stage, apoptosis, cytotoxicity and ultimately cell death (2).

Doxorubicin is an anthracycline type of antibiotic. All anthracyclines can intercalate and interfere with DNA and RNA synthesis. Doxorubicin is the most active agent against solid tumours, particularly breast cancer. In patients with advanced breast cancer, docetaxel and doxorubicin have the greatest activity as single agents (3).

Cyclophosphamide, also known as cytophosphane, is a nitrogen mustard alkylating agent from the oxazophoridine group. Cyclophosphamide is used to treat various types of cancer and some autoimmune disorders by slowing-down or stopping cell growth (4). Cyclophosphamide, along with other chemotherapy agents is used for the treatment of lymphomas, some forms of leukaemia, and some types of solid tumours.

Considering the chemotherapeutic activity of cyclophosphamide as a pro-drug that is converted to active forms in the liver, we attempted to evaluate the possible effect of the addition of cyclophosphamide to doxorubicin and docetaxel on the metabolic activity of the MCF-7 breast cancer cell line.

## Materials and Methods

*Cell culture.* The cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). It is a model cell line of human mammary carcinoma exhibiting features of differentiated mammary epithelium (5). Cells were cultured in 10% Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, California, USA) containing foetal bovine serum (FBS, Invitrogen), mixture of penicillin and streptomycin (Pen Strep) (Invitrogen), with

*Correspondence to:* Dr. Marianna Trebunova, Department of Medical and Clinical Biophysics, Faculty of Medicine, P.J. Safarik University, Trieda SNP 1, Kosice, 040 11, Slovakia. E-mail: marianna.trebunova@upjs.sk

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5% CO<sub>2</sub> and 95% humidity at 37°C. The solutions used were of the cell culture grade quality and the equipment used in the cell culture was commercially pre-sterilized and disposable.

**Drugs.** Docetaxel, doxorubicin and cyclophosphamide were kindly provided by Dr. Andrašin (UPJS Kosice, Faculty of Medicine, Department of Oncology). Docetaxel was stored at room temperature in polysorbate 80 as 40 mg/ml concentrate. Doxorubicin was stored as 3.4 mM stock solution in distilled water at room temperature. Cyclophosphamide was stored as 213.8 mg cyclophosphamide monohydrate, equivalent to 200 mg cyclophosphamide, at room temperature. The solutions of cytostatics were vigorously stirred before dilution in the filtered cell culture medium and stored at -20°C. Cytostatics were directly applied into the culture medium to obtain 40 nM, 60 nM and 120 nM docetaxel in the first set of experiments and 200 nM, 500 nM and 1000 nM doxorubicin in the subsequent one. Finally, we used 600 nM cyclophosphamide combined with the mixture of docetaxel and doxorubicin.

**Viable cell count and test.** Antiproliferative effects of docetaxel, doxorubicin and cyclophosphamide on parental MCF-7 cells were measured using standard colorimetric test described in detail in the study of Sabo (6). Briefly, cell suspensions containing 4×10<sup>4</sup> viable cells were cultivated in 96-well tissue culture plates (Sarstedt, Nuembrecht, Germany) with and without tested drugs in a final volume of 100 µl, as duplicates. Drugs were added approximately 24 h after seeding. Cells were allowed to grow for 24 h after the drug treatment. The optical density of each well was measured spectrophotometrically at 480 nm in an MRX Dynatech ELISA reader (UK). The obtained values were also calculated and expressed as a percentage of metabolic activity in comparison with controls considered to have 100% metabolic activity. Cells were stained with trypan blue (Sigma Aldrich, St. Louis, Missouri, USA) with a 1:9 ratio of trypan blue to cell suspension. Cells were counted in a Burker counting chamber under light microscopy. Dark blue cells were evaluated as dead cells. To construct the growth curves, cells were observed for 11 days with cell number's counted every 24 h. The metabolic activity of the cells for the combination of the docetaxel/doxorubicin/cyclophosphamide was evaluated on the fifth day of the cell growth.

**Statistical analysis.** The results for the growth curves are presented as the mean ± standard error of the mean (SEM). For the metabolic activity assessed by the MTT test, standard deviation (SD) is used. Comparisons between groups were analysed using the t-test (two-sided). Probability values of *p*<0.05 were considered statistically significant.

## Results

The cell count data were expressed as the number of cells in 1 ml and subsequently the growth curves for the parental untreated MCF-7 and drug-applied sublines were drawn. From the exponential phase of the growth curves, a linear growth plot was generated. The slope of the trend lines gives the growth rate,  $\mu$ , that was used to calculate the doubling time,  $t_d$ , as follows:

$$t_d = \frac{\ln 2}{\mu}$$

Since counts were performed five times for each experiment, the mean values for  $t_d$  calculated from the slopes of the trend lines with the corresponding SEM for *n*=5, were considered.

In Figures 1 and 2, growth curves for docetaxel- and doxorubicin-treated MCF-7 cells are presented in comparison with those for the parental cells. The MCF-7 cells became resistant to docetaxel and doxorubicin by increasing the concentration up to 120 nM and 1000 nM, respectively. Application of docetaxel slowed the growth of the cell population over the whole concentration range, as shown in Figure 3. The doubling time  $t_d$  for MCF-7 cells, treated with the concentration of 120 nM docetaxel, extended to approximately 37 hours, almost 40% more than the doubling time for the untreated cell population. According to the statistical analysis, the change in the growth rate was significant (*p*<0.05) for MCF-7 cells treated with 120 nM docetaxel in comparison with untreated MCF-7 cells and for MCF-7 cells treated with 120 nM docetaxel in comparison with MCF-7 cells treated with 40 nM docetaxel (Table I).

Concerning the effect of doxorubicin, the application of the drug up to 500 nM slowed the growth of cells, increasing the doubling time nearly two-fold in comparison with the parental line. Doxorubicin at 1000 nM surprisingly shortened the doubling time in comparison with 500 nM of doxorubicin. According to the statistical analysis, the change in the growth rate was significant (*p*<0.05) for the whole concentration range from 200 nM to 1000 nM of doxorubicin applied sublines, in comparison to sensitive MCF-7 (Table II).

In Figure 3, the metabolic activity of MCF-7 cells according to docetaxel treatment and doxorubicin treatment is depicted with highlighted significant difference between the parental MCF-7 cells, and cells after the treatment with have a cytostatics. It is clear that 40 and 60 nM docetaxel did not significant effect on the metabolic activity of cells. Therefore, it was interesting to investigate the ability of cyclophosphamide to potentiate the effect of docetaxel and of doxorubicin on the metabolic activity of the MCF-7 cell line. Cyclophosphamide at 600 nM was added directly into the filtered cell media containing docetaxel and doxorubicin. The results of MTT tests are presented in Figure 4. The combination of 40 nM docetaxel/200 nM doxorubicin/600 nM cyclophosphamide did not significantly change the metabolic activity of cells, although 200 nM doxorubicin by itself reduced the cell growth. Increasing docetaxel to 60 nM but using the same levels of doxorubicin and cyclophosphamide resulted in the significant decrease in metabolic activity, although 60 nM docetaxel by itself had no effect. The same is valid for the combination with 500 nM doxorubicin. Cyclophosphamide clearly potentiates the effect of docetaxel. The fact that combination of 60 nM docetaxel/200 nM doxorubicin/600 nM cyclophosphamide triggers changes in metabolic activity, is of great interest, considering that docetaxel must be used at a minimal

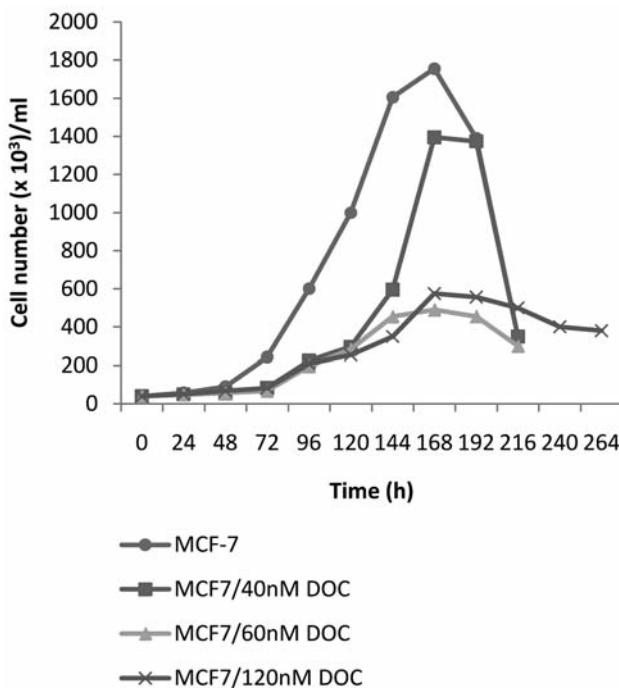


Figure 1. Growth curves of docetaxel (DOC)-treated MCF-7 cells.

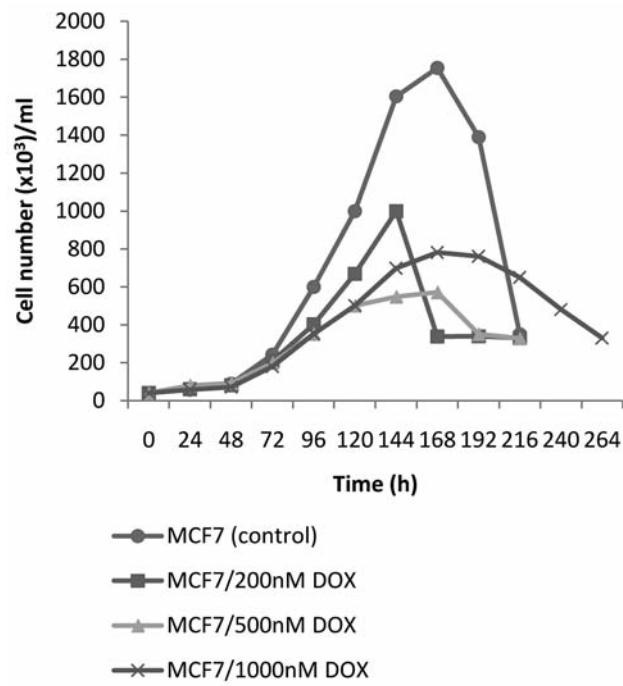


Figure 2. Growth curves of doxorubicin (DOX)-treated MCF-7 cells.

concentration of 120 nM to retard such activity if applied separately ( $p<0.05$ ). Thus, cyclophosphamide at 600 nM concentration seems to enhance the influence of docetaxel when combined with doxorubicin.

The effect of cyclophosphamide was statistically analyzed regarding doubling times of MCF-7 cells treated with combinations of docetaxel/doxorubicin in comparison with doubling times of MCF-7 cells treated with combinations of docetaxel/doxorubicin/cyclophosphamide (Table III). According to statistical data, no significant change ( $p>0.05$ ) was observed for combinations of docetaxel/ doxorubicin in comparison with the same combinations of cytostatics with cyclophosphamide when the concentration of combinations docetaxel/doxorubicin was the same in both groups. A significant change ( $p<0.01$ ) was observed only when the concentration of docetaxel was raised to 60 nM and doxorubicin to 500 nM compared to the combination of 40 nM doxorubicin/200 nM docetaxel/600 nM cyclophosphamide. A significant change ( $p<0.05$ ) was also observed when the concentration of docetaxel and doxorubicin was not raised compared to the combination of 60 nM doxorubicin/200 nM docetaxel/600 nM cyclophosphamide or concentration of docetaxel was raised to 500 nM. Metabolic activity of MCF-7 cells was also significantly altered ( $p<0.05$ ) after the treatment with the combination of 60 nM doxorubicin/500 nM docetaxel/600 nM cyclophosphamide in comparison with combinations C1, C2 and C3.

Table I. Statistical analysis of doubling time ( $t_d$ ) values for docetaxel-treated MCF-7 cells.

	MCF-7	40 nM DOC	60 nM DOC	120 nM DOC
MCF-7		$p>0.05$	$p>0.05$	$p<0.05^*$
40 nM DOC			$p>0.05$	$p<0.05^*$
60 nM DOC				$p>0.05$

\*Represents significant difference in  $t_d$  between groups of cells.

Table II. Statistical analysis of  $t_d$  values for doxorubicin-treated MCF-7 cells.

	MCF-7	200 nM DOX	500 nM DOX	1000 nM DOX
MCF-7		$p<0.05^*$	$p<0.05^*$	$p<0.05^*$
200 nM DOX			$p<0.01^*$	$p<0.01^*$
500 nM DOX				$p<0.01^*$

\*Represents significant difference in  $t_d$  between groups of cells.

## Discussion

For years, there has been a considerable interest in the employment of combinations of drugs in the treatment of breast cancer (7-9). In accordance with this idea, a therapeutic effect is achieved if the drugs in combination

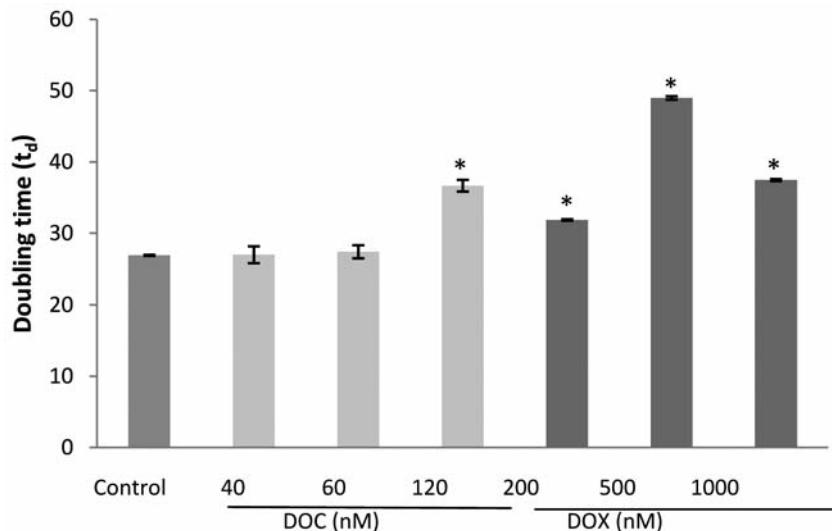


Figure 3. Population doubling time  $t_d$  for docetaxel (DOC)- and doxorubicin (DOX)-treated MCF-7 cells. \*Significantly different from the control at  $p<0.05$ .

produce an effect greater than the sum of their individual effects and provide a more effective therapy.

MCF-7 cells are extensively used as a cell model to investigate the application of antitumour drugs. Several studies (10-16) evaluated antiproliferative effects of different drugs on resistant cells. İseri (17) reported an effort to develop docetaxel- and doxorubicin-resistant MCF-7 human breast carcinoma sublines by stepwise selection of resistant cells, and the antiproliferative efficacy of combined drug applications on resistant cells. In that study, it was shown that combination of doxorubicin and docetaxel resulted in a significant synergistic antiproliferative effect on the docetaxel-resistant cells, whereas tamoxifen exerted an additive antiproliferative effect with docetaxel. Furthermore, combinations of paclitaxel, docetaxel and tamoxifen with doxorubicin exerted significant synergistic effects on doxorubicin-resistant cells. Thus, according to previous findings, tamoxifen can be used in combination with both docetaxel and doxorubicin. In our study, we confirmed the finding that docetaxel and doxorubicin-treated MCF-7 cell lines developed varying degrees of resistance to selective drugs. The degree of resistance increased with the drug concentration used.

A synergistic activity has also been shown for cyclophosphamide combined with ifosfamide (18). These alkylating agents have also been combined with other anticancer agents. As a pilot study to the proteomic research of MCF-7 cells treated with the anticancer drug-combination docetaxel/ doxorubicin/cyclophosphamide, we investigated the effect of cyclophosphamide on the metabolic activity of docetaxel/ doxorubicin-treated MCF-

Table III. Statistical analysis of  $t_d$  values for MCF-7 cells treated with combinations of cytostatics with and without cyclophosphamide. Statistical significance was assessed using t-test. Concentration of drugs in the mixture: C1: DOC=40 nM, DOX=200 nM; C2: DOC=60 nM, DOX=200 nM; C3: DOC=40 nM, DOX=500 nM; C4: DOC=60 nM, DOX=500 nM

	C1, with CPA	C2, with CPA	C3, with CPA	C4, with CPA
C1, no CPA	$p>0.05$	$p<0.05^*$	$p>0.05$	$p<0.05^*$
C2, no CPA	$p<0.01^*$	$p>0.05$	$p>0.05$	$p<0.05^*$
C3, no CPA	$p>0.05$	$p<0.05^*$	$p>0.05$	$p<0.05^*$
C4, no CPA	$p<0.01^*$	$p>0.05$	$p>0.05$	$p>0.05$

\*Represents significant difference in  $t_d$  between groups of cells.

7 cells. The effectiveness of cyclophosphamide to enhance the effect of antiproliferative drugs has been demonstrated by Singh *et al.* (19). The authors tried to overcome the limited clinical application of cyclophosphamide as a breast cancer therapy by combining 5 mM of the drug with resveratrol. They showed that was due to caspase-mediated cytotoxic activity of cyclophosphamide on MCF-7 cells. The high concentration of cyclophosphamide (500 µg/ml) in combination with doxorubicin and 5-fluorouracil was used by Kugawa *et al.* (21) to evaluate the features of cell death induced *in vitro* using the human breast cancer cell line MCF-7. They suggested that multi-drug administration causes both of apoptotic and non-apoptotic, but it becomes completely non-apoptotic death.

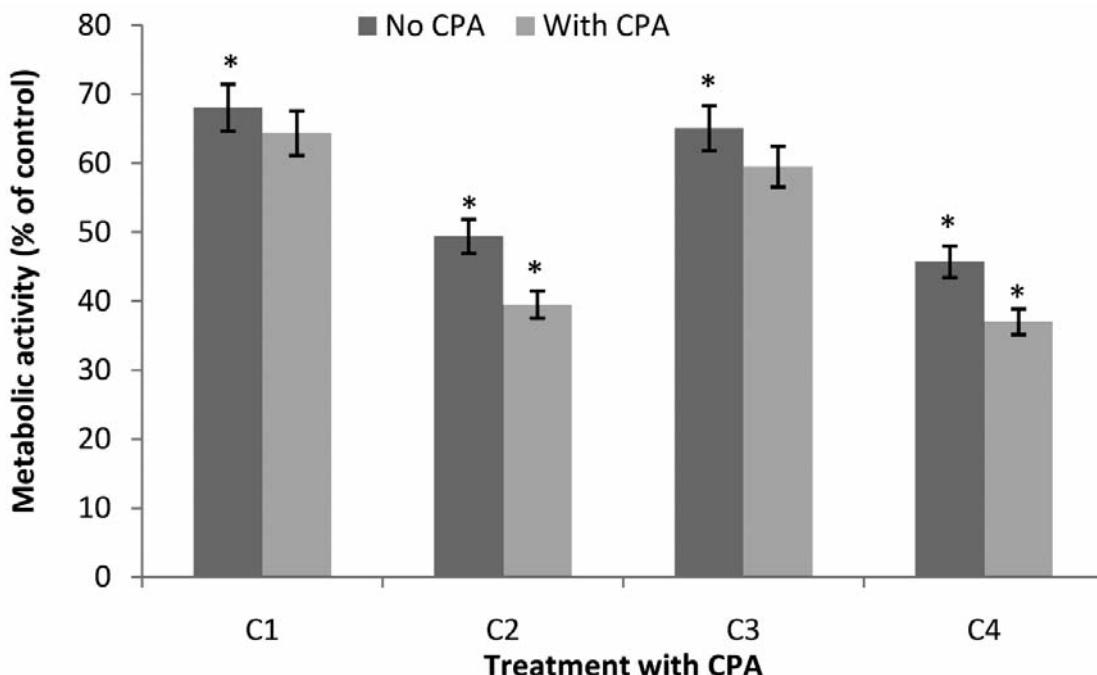


Figure 4. The effect of cyclophosphamide on the potency of the mixture of docetaxel and doxorubicin. Bars represent the mean $\pm$ SD of four independent experiments. Statistical significance was assessed using t-test. \* $p < 0.05$ . Concentration of drugs in the mixture: C1: DOC=40 nM, DOX=200 nM; C2: DOC=60 nM, DOX=200 nM; C3: DOC=40 nM, DOX=500 nM; C4: DOC=60 nM, DOX=500 nM.

Acute toxicity of cyclophosphamide has been well documented by large clinical trial groups (22). As the therapeutic effectiveness of cyclophosphamide is limited by the high-dose toxicity, in our study we tried to reduce the concentration to the lowest possible level (600 nM). Some significant changes were observed in metabolic activity of cells treated with docetaxel/doxorubicin/cyclophosphamide in comparison with control, namely, increasing concentration of docetaxel to 60 nM but using the same level of doxorubicin resulted in a significant decrease in metabolic activity, although 60 nM docetaxel by itself had no effect. Likewise, the combinations of 60 nM docetaxel/500 nM doxorubicin with cyclophosphamide were able to reduce the cell growth significantly.

However, the potential of cyclophosphamide to enhance the influence of docetaxel when combined with doxorubicin does not seem to be well proven when comparing doubling times of MCF-7 cells treated with combinations of docetaxel/doxorubicin with cells treated simultaneously with cyclophosphamide. There were no significant changes between these two groups, when the concentration of docetaxel/doxorubicin was the same in both groups. Only some preferred combinations were superior, e.g. 40 nM docetaxel/200 nM doxorubicin with cyclophosphamide to 60 nM docetaxel/200 nM doxorubicin or 60 nM

docetaxel/500 nM doxorubicin; 60 nM docetaxel/200 nM doxorubicin with cyclophosphamide to 40 nM docetaxel/200 nM doxorubicin or 40 nM docetaxel/500 nM doxorubicin.

These results suggest that nanomolar concentrations of cyclophosphamide show an ambiguous effect on MCF-7 cells *in vitro*. The possibility of a new chemotherapeutic combination regimen leading to improvements in the treatment of breast cancer require to re-value the concentration of cyclophosphamide and understand the molecular mechanism of cell-killing by combinations of docetaxel/doxorubicin/cyclophosphamide.

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