

**DRUG BIOCHEMISTRY****INFLUENCE OF VITAMINS C AND E ON CYTOTOXIC ACTIVITY  
OF ADRIAMYCIN IN CHOSEN CELL CULTURES**

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**Abstract:** Influence of different concentrations of ascorbic acid (vitamin C) and dl- $\alpha$ -tocopherol acetate (vitamin E) on *in vitro* cytotoxic adriamycin activity, in: embryonic human fibroblasts (CLV102), human melanoma cells (ME18) and adriamycin-resistant subline cells (ME18/R), was studied.  $IC_{50}$  value for each compound (compound concentration in the culture medium, for which 50% of cells survive) was determined. Cells' survival after the used agent was examined with trypan blue test. The relationship between different concentrations of vitamin C and toxicity of adriamycin, used at appropriate  $IC_{50}$  concentrations, was expressed for all the examined cells as their survival decrease, being in direct proportion to the concentration increase of this vitamin in the medium. In the case of influence of vitamin E on adriamycin cytotoxicity, the protective effect of this vitamin was observed in the concentration range: from 5 to 300  $\mu\text{g/ml}$  ( $p \leq 0.0001$ ), as an increase of the examined cell survival for ME18, ME18/R as well as for CLV102, comparing to the control ( $p=0.05$ ) without this vitamin. Paralelly, a statistically significant survival decrease was observed, if the concentration of vitamin E in the culture medium exceeded 500  $\mu\text{g/ml}$ . Received results showed different, in defined concentration range, effects of the vitamin C or vitamin E activity for adriamycin cytotoxicity. These effects were similar for all the examined cells.

**Keywords:** adriamycin, vitamin C, vitamin E, cytotoxicity.

Adriamycin (ADR) is an antibiotic from anthracycline group with a wide spectrum of activity, used commonly in anticancer therapy. It is used as well in the treatment of solid tumours of: breast, ovary, lung as in leukaemias. Its cytotoxicity is connected with the multidirectional activity for genetic cell material (DNA crosslinks formation, intercalation to DNA, stabilization of cleavable complex topo II–DNA) and free radicals generation (1,2).

The cardiotoxicity and inhibition of bone marrow activity are very important problems, the anthracycline chemotherapy struggles with. Adriamycin cardiotoxic activity is probably connected with the reaction of oxygen radicals with the cardiac cells' membranes. The microscopic examination of cardiac tissue after adriamycin administration, demonstrated serious mitochondrial membrane injuries (3).

Additionally, low level of catalase activity (an antioxidant enzyme) is characteristic for cardiac muscle cells (4).

It was found that free oxygen radicals affect the cells by oxidising unsaturated fatty acids, among others, causing the membrane destruction and the calcium transport modification (5). As far as mitochondrial and nuclear DNA, is concer-

ned, free radicals may cause the mutation even in far organs of the organism. Potentially, this phenomenon may allow to create primary neoplastic changes.

Adriamycin cytotoxicity, depended on free radicals generation in patients treated antineoplastically, causes negative effects in the shape of acute and chronic cardiac muscle inflammation (3,6,7). At a long – term chemotherapy, it leads to the circulatory insufficiency, dangerous for the patient, and it limits adriamycin usage in anticancer therapy. On the basis of the literature, it is known that the use of combined therapy with adriamycin, vitamin E or C, partially abolishes the toxic effect of this drug (used alone) exerted on the cardiac muscle (8,9,10,11). Both vitamins show oxidative – reductive properties and belong to the compounds called „antioxidants” or free oxygen radicals „scavengers” (12,13). In the  $\alpha$ -tocopherol *via*  $\alpha$ -tocopherol radical oxidation reaction, biologically inactive tocopherolochinon is formed. The  $\alpha$ -tocopherol oxidation, as mentioned above, is the reversible reaction. It is supposed that ascorbic acid takes part in tocopherol regeneration, taking away the oxygen from the tocopherol radical and changing itself into the reversibly oxidised form – dehydroascorbic acid (14). Nowadays, it is currently

assumed, that vitamin E is the main, fat-soluble antioxidant, protecting the cell membrane phospholipids against the oxidation (15,16). The co-operation of vitamin E and antioxidant enzymes as: superoxide dismutase and glutathione peroxidase, was experimentally proved (17).

Recently, a paper was issued, pointing to another mechanism of vitamin C antioxidant activity i.e., including the activation of repair enzymes, taking part in excision of oxidised purine bases e.g.: 8-oxodesoxyguanosine (18).

The aim of our work was to examine the influence of vitamin C and vitamin E on the cytotoxic adriamycin activity in human normal and neoplastic cells differing in sensitivity to this drug.

## EXPERIMENTAL

### Chemical reagents

Adriamycin – doxorubicin (ADR) (Farmitalia), ascorbic acid – vitamin C (Sigma), dl- $\alpha$ -tocopherol acetate – vitamin E (Sigma), Eagle's medium – MEM (Gibco), calf fetal serum (Bioproduct), dimethylsulfoxide – DMSO (Merck), trypan blue (BDH Chemical Ltd.).

### Cell cultures

Experiments were led with the use of normal embryonic human fibroblasts (CLV102), human melanoma cells (ME18), and experimentally obtained subline of human melanoma, resistant to adriamycin (ME18/R).

The method of resistant subline acquisition was described in earlier work (19). Cells were cultured in MEM medium, enriched with 10% of fetal calf serum and antibiotics (penicillin 100 i.u./ml and streptomycin 100  $\mu$ g/ml), in a 5% CO<sub>2</sub> atmosphere at 37°C.

CLV102 cells were obtained from Central Laboratory for Sera and Vaccines in Warsaw, ME18 cells were obtained from Oncology Institute in Warsaw.

The cell cultures (at a density of about 10<sup>5</sup> cells/ml), were incubated at 37°C and 5% CO<sub>2</sub> in Nunc flasks (25 cm<sup>2</sup>) for 24 h. After that time, the medium with adriamycin, vitamin C or vitamin E was added and the incubation was continued for another 24 hours.

ADR was used in the concentration range from 0.125  $\mu$ g/ml to 40  $\mu$ g/ml, vitamin C: from 12.5  $\mu$ g/ml to 400  $\mu$ g/ml, and vitamin E: from 0.2  $\mu$ g/ml to 600  $\mu$ g/ml. The primary adriamycin and vitamin C solutions were prepared in water, vitamin E was dissolved in DMSO (the maximal final DMSO concentration in the medium was 0.2%, and was not cytotoxic).

After 24 h of incubation of the cell cultures with the examined agents, the media were removed, the cells were rinsed with PBS, trypsinized, stained with trypan blue and then, the rate of living cells was determined. On the basis of the results obtained, the values of IC<sub>50</sub> were expressed graphically.

### Assessment of the influence of vitamin C or vitamin E on cytotoxic adriamycin activity

To examine the influence of vitamin C or vitamin E on adriamycin cytotoxicity, the cell cultures were used as described above. After 24 h of incubation at 37°C and a 5% CO<sub>2</sub> atmosphere, the media with ADR, used at the IC<sub>50</sub> appropriate concentration, were added. Paralelly, vitamin C used in the concentration range from 12.5  $\mu$ g/ml to 400  $\mu$ g/ml, or vitamin E: from 0.2  $\mu$ g/ml to 600  $\mu$ g/ml were added to the media. The cell cultures treated with ADR only were the control ones.

### The influence of vitamin E on cytotoxic effect of the combined administration of adriamycin and vitamin C

In the following experiments the protective influence of vitamin E on ME18 cell cultures was determined. There were added to the medium simultaneously: the IC<sub>50</sub> dose of adriamycin and vitamin C at a concentration which did not cause toxic effect, however, it increased the adriamycin-induced cytotoxic effect. Vitamin E was added in the concentration range from 25  $\mu$ g/ml to 200  $\mu$ g/ml. Examination was executed with the use of ME18 cells only, because the effects obtained with the normal and neoplastic cells were similar.

## RESULTS AND DISCUSSION

In the present work, the influence of both, commonly known as antioxidants, vitamins C and E on adriamycin cytotoxic activity (cytostatic, whose mechanism of activity is possibly connected with the free oxygen radicals generation) was studied.

The oxidising activity of free radicals causes, that the therapeutic adriamycin activity is disturbed with side effects e.g. cardiotoxicity. In this study, human melanoma cell cultures (ME18 and ME18/R), differing in sensitivity to adriamycin and human embryonic fibroblasts cultures, (CLV102), were used.

Table 1 shows the results of adriamycin, vitamin C and vitamin E cytotoxic activity in the chosen cell cultures.

The IC<sub>50</sub> value for adriamycin in CLV102 and ME18 cell cultures was 1  $\mu$ g/ml, ME18/R cells have

decreased the sensitivity to adriamycin: the  $IC_{50}$  value in this case was 10  $\mu\text{g/ml}$ . Sensitivity of the chosen cell cultures to vitamin C activity was determined in the concentration range up to 400  $\mu\text{g/ml}$ . CLV102 cells distinguished by increased sensitivity to ascorbic acid – the  $IC_{50}$  value was 200  $\mu\text{g/ml}$ .

The vitamin E toxicity was, however, the same for all the cell cultures used:  $IC_{50}$  value was 500  $\mu\text{g/ml}$ .

Figure 1 shows the influence of vitamin C on the adriamycin cytotoxic activity. Vitamin C caused a proportional increase of adriamycin cytotoxicity in all the examined cell cultures.

The strongest cytotoxic effect of the combined adriamycin and vitamin C administration was observed for embryonic human fibroblasts, CLV102. The course of survival curves for human melanoma cells, sensitive and resistant to adriamycin (ME18; ME18/R), was similar. These results correlate well with the sensitivity of the cells to vitamin C. This correlation is especially visible for the concentration exceeding 100  $\mu\text{g/ml}$ . The significant decrease of CLV102 cells survival was then observed. So, the literature data (9,12,13), concerning protective activity of vitamin C towards adriamycin, which should be expressed as diminished cytotoxic activity, were not confirmed. However, it should be taken into consideration that most of works suggesting the protective vitamin C activity towards adriamycin was examined *in vivo*, with the use of animals and this may be the reason of the discrepancy observed.

On the basis of literature, it is known that vitamin C, studied *in vivo*, prolongs the life time of experimental animals with cancer (9,12,14).

It is known too that vitamin C in large doses and in the presence of iron and copper ions, which are present in the culture medium, may act as oxidant (20) causing an increase in the adriamycin toxicity.

Table 1.  $IC_{50}$  value, determined graphically for: adriamycin, vitamin C and vitamin E for the tested cell cultures.

Cells	$IC_{50}$ [ $\mu\text{g/ml}$ ]		
	Adriamycin	Vitamin C	Vitamin E
CLV102	$1 \pm 0.16^*$	$200 \pm 13.36$	$500 \pm 31.18$
ME18	$1 \pm 0.19$	$373 \pm 11.70$	$500 \pm 26.31$
ME18/R	$10 \pm 0.91$	$310 \pm 10.00$	$500 \pm 32.99$

\* average value from five following experiments  $\pm$  SD

Vitamin C concentrations in the culture medium, 100 – 400  $\mu\text{g/ml}$ , used in this work, necessary to obtain significant effects *in vitro*, were ten times higher than the value taken as a normal vitamin C concentration in the serum (50  $\mu\text{mol/l}$ ) (21,22).

Figure 2 shows the influence of vitamin E on the adriamycin cytotoxic activity.

The protective effect of vitamin E was obtained in the concentration range from 5 to 300  $\mu\text{g/ml}$  ( $p \leq 0,0001$ ). It was expressed as an increase

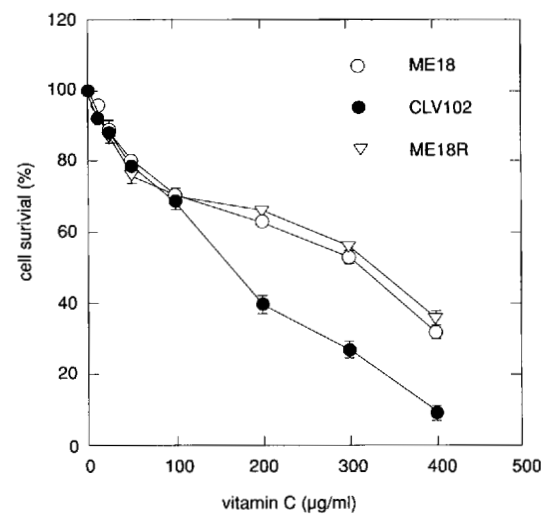


Figure 1. Influence of vitamin C on adriamycin cytotoxic activity in chosen cell cultures. Adriamycin was added to the culture medium at the concentration  $IC_{50}$ .

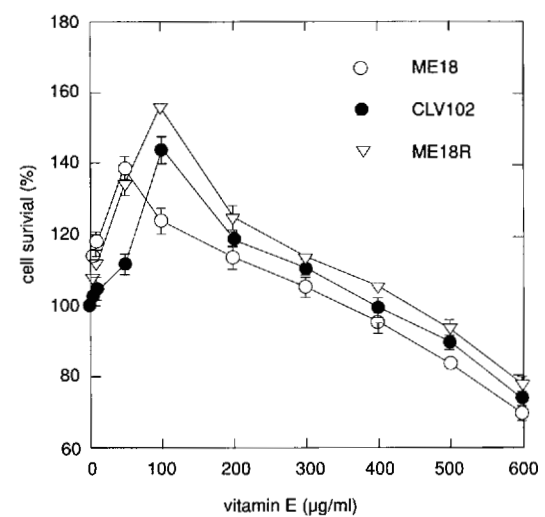


Figure 2. Influence of vitamin E on adriamycin cytotoxic activity in chosen cell cultures. Adriamycin was added to the culture medium at the concentration  $IC_{50}$ .

Table 2. Influence of vitamin E on the survival of ME18 cells after combined administration of adriamycin and vitamin C.

Experimental arrangement	Living cells rate
Control	100.0
Vitamin C*	98.2 ± 3.5***
ADR**	48.1 ± 1.7
ADR + Vitamin C	39.8 ± 2.7
ADR + Vitamin C + Vitamin E 25 µg/ml	54.0 ± 2.1
50 µg/ml	88.8 ± 2.1
100 µg/ml	101.8 ± 4.3
200 µg/ml	98.3 ± 5.2

\* 50 µg/ml

\*\* 1 µg/ml

\*\*\* average value from three following experiments ± SD

of the rate of living cells exceeding the control value for both neoplastic ME18 and ME18/R cells and normal human cells CLV102. The most significant protective effect of vitamin E was obtained at a concentration 100 µg/ml. For the vitamin E concentration – 400 µg/ml, the cell survival was approximate to the control level. Simultaneously, statistically significant decreases of cell survival ( $p \leq 0.001$ ) for the vitamin E concentration 500 µg/ml and higher were observed.

These results are compatible with those obtained by other authors studying the protective, antioxidant vitamin E activity (10,11,12,16,17).

On the basis of the current results it may be suggested that vitamin E, in some range of concentrations, could abolish the toxic adriamycin activity. In our experiments vitamin E, however, increased the survival of both embryonic fibroblasts CLV102 and neoplastic ME18 and ME18/R cells. Differences in the response to vitamin E activity of these cells are dependent on the sensitivity to adriamycin and were not observed. Similar results of vitamin E activity were obtained *in vivo* in the case of hepatocellular carcinoma (23,24).

Table 2 shows the results concerning the influence of vitamin E on the cytotoxic effect of combined adriamycin and vitamin C administration. The protective effect of vitamin E was dose-dependent in the range of vitamin E concentrations from 25 µg/ml to 100 µg/ml. Increase of vitamin E concentration to 200 µg/ml showed insignificant influence on the rate of living ME18 cells.

The vitamin E concentration – 100 µg/ml, as in the previous experiment, was the optimal one. Vitamin E concentration 100 µg/ml in the culture

medium equals the dose of about 7 g for the adult person with the body weight of 70 kg. Vitamin E gram doses are used in prophylaxis of myocardial ischaemia (22,25,26) as well as in prophylaxis of neoplastic diseases and also in supporting in these therapies (27,28).

If the main role of vitamin C is the regeneration of tocopherol radical, formed in the oxygen radical scavenging (29), it is possible that in such low doses, vitamin C could fulfill its protective function towards vitamin E, being at the same time non toxic. The solution of the problem concerning the level of used doses of both vitamins needs further studies.

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