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Enhancement of cytotoxicity and DNA binding of cisplatin in Dalton's lymphoma cells by α-tocopherol

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A combination treatment of cisplatin and α-tocopherol on Dalton's lymphoma cells *in vitro* was studied in order to examine the effect of α-tocopherol on cisplatin cytotoxicity and binding of platinum to DNA (DNA platination) or its removal from DNA. Cisplatin cytotoxicity which is measured as cell survival of Dalton's lymphoma cells was found to be enhanced by various factors, including concentration of drug, treatment duration and presence of α-tocopherol in the medium. Tumour cells treated with increasing concentrations of cisplatin or cisplatin with α-tocopherol lower cell survival and increase DNA platination. There was a significant correlation between percentage survival of Dalton's lymphoma cells and DNA platination. The study of percentage removal of platinum has shown an inverse linear correlation between DNA damage and DNA repair. The enhanced cytotoxicity of cisplatin by α-tocopherol could be attributed to increased platinum binding to DNA, which might decrease DNA repair.

Keywords: Cisplatin, cytotoxicity, Dalton's lymphoma, DNA platination, α-tocopherol.

CISPLATIN is successfully used in the chemotherapy of a wide variety of experimental malignant tumours[1–3] and also in the treatment of many human cancers[4,5]. Many reports have been published describing the intercalation of cisplatin with DNA[6–8], which suggest that cellular DNA could be the primary target for cisplatin in its cytotoxicity. The antitumour activity of cisplatin is believed to result from the interaction of drug with DNA, which leads to the formation of different types of adducts through reaction of the bifunctional platinum compound with N^7 atoms of the nucleobases guanine and adenine. The major adducts are intrastrand crosslinks formed by the binding of cisplatin on two neighbouring guanines, on adenine and guanine and on two guanines separated by one or more nucleobases. Other types of adducts formed are the interstrand crosslinks on two guanines and monofunctionally bound cisplatin on guanines[8–10]. Hyperthermia enhances the cytotoxic effect of cisplatin in both cisplatin-sensitive and cisplatin-resistant mammalian cell lines[11–13]. The enhanced cytotoxicity may be due to increased DNA crosslinking by cisplatin, increased cellular accumulation of platinum or decreased repair of drug-induced DNA damage[12–14]. The amount of platinum bound to DNA is also likely to be related to the sensitivity of cells to cisplatin[15]. Prasad and Rama[16] showed that vitamin E induced both morphological differentiation and growth inhibition of murine and human neuroblastoma cells in culture at least in part by an antioxidant mechanism. Vitamin E probably inhibits DNA synthesis in tumour cells because it inhibits cell division. It may act similarly at the site on RNA or DNA where cisplatin binds[17]. Prasad *et al.*[17] demonstrated the presence of vitamin E-binding proteins in tumour cells in culture, but the relationship of vitamin E-binding proteins to the mechanism of action of vitamin E is unknown. The association of a significant amount of radioactive vitamin E with the purified chromatin suggests that vitamin E may modulate genetic expression in mammalian cells[17]. The present study was undertaken to investigate the effects of vitamin E on cisplatin cytotoxicity and on binding of platinum to DNA (DNA platination) or its removal from DNA.

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C3H/He strain of syngeneic mice was obtained from Chittaranjan National Cancer Research Centre, Kolkata, India and maintained in the laboratory. Mice were randomly distributed in polypropylene cages and fed on a commercial diet (Goldmohar, Lipton, India) and tap water *ad libitum*. Mice were kept under standard condition (specifically pathogen-free, temperature ranging from 22°C to 23°C and relative humidity 65–70%). Mice, both males and females, 8–10-week-old and weighing 20–22 g were used in the present study. Transplantable ascites Dalton's lymphoma was obtained from Chittaranjan National Cancer Research Centre, and maintained in the laboratory by regular serial transplantations.

For cytotoxicity assay *in vitro*, Dalton's lymphoma cells were plated at high density (8×10^7 cells/dish) at time 0 in DMEM containing 10% foetal calf serum, 10 mM NaHCO$_3$, 0.3% glutamine, antibiotics and different concentrations of either cisplatin, α-tocopherol or a combination of both and incubated for 30, 60, 90, 120, 150 and 180 min. Control dishes were treated with equal amounts of DMSO used as a solvent for α-tocopherol. At the end of the drug treatment, the medium containing drugs was aspirated-off, cells were washed with isotonic PBS, resuspended in arginine-deficient MEM (ADM) with dialysed foetal bovine serum (DFBS) and incubated for 72 h at 37°C. After incubation cells were trypsinized and viable cells were counted by trypan blue exclusion test. Each assay was repeated thrice and the results were analysed by Student's *t* test.

Stationary phase cultures of Dalton's lymphoma cells were exposed to either cisplatin or α-tocopherol or a combination of both for 120 min. Cells were harvested either immediately after incubation for the estimation of

platinum-bound to DNA (DNA damage) or washed with isotonic phosphate buffer saline and further incubated at 37°C for 24 h in ADM containing 2.5% DFBS without any drug and harvested for DNA repair.

DNA was isolated according to Grimaldi *et al.*[18]. Cells were harvested by centrifugation at 1000 *g* for 10 min and washed with isotonic phosphate buffer saline. Cells were lysed with lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl and 1% w/v SDS). Lysate was incubated with proteinase K at 37°C for 12 h to digest protein. DNA was extracted by mixing equal volume of phenol saturated with 10 mM Tris-HCl, 1 mM EDTA and 0.1 M NaCl (pH 7.6). After phenol/chloroform extraction (×2) DNA solution was treated with 10 µg/ml RNAaseT$_1$ at 37°C for 1 h followed by extraction with equal amount of phenol/chloroform (×2) and chloroform (×1). DNA was precipitated with 1/10 volume of sodium acetate (3 M) and 2 volumes of cold absolute ethanol. Precipitated DNA was washed twice with 70% ethanol and dissolved in 0.1 M nitric acid. Platinum bound to DNA was estimated by ICPMS as described by Tothill *et al.*[19] using H$_2$PtCl$_6$ in 5% HCl as a standard.

The present investigation has shown the effects of cisplatin at the cellular level through a study of its lethal activity in an *in vitro* system. Results obtained in the present study with Dalton's lymphoma cells are expressions of cell killing assay and not the growth inhibition assay. It was found that cytotoxicity of cisplatin depends upon its concentration in the culture media. Survival of Dalton's lymphoma cells treated *in vitro* with different concentrations of cisplatin for a fixed time duration of 1 h is shown in Figure 1. There exists an inverse linear correlation between the concentration of cisplatin in the medium and % survival of lymphoma cells. IC$_{50}$ of cisplatin was found to be 17.5 µg/ml (Figure 1). Figure 2 shows the effect of treatment duration on cell survival after incubation with different concentrations of cisplatin. The percentage survival of Dalton's lymphoma cells decreases with increase in treatment duration. Tumour cells showed 63–73% survival when incubated with 5–10 µg/ml cisplatin for 60 min; however, it decreased to 25–46% when incubation time was increased to 120 min. High doses (15–30 µg/ml) of cisplatin decreased the percentage survival to less than 10% after 120 min treatment duration. Thirty µg/ml cisplatin has shown less than 10% survival, even after 60 min treatment duration (Figure 2). Survival of lymphoma cells incubated with different concentrations of α-tocopherol for various time periods was not significantly different from control (Figure 3). DMSO was not found to be cytotoxic to Dalton's lymphoma cells *in vitro*. Combination of α-tocopherol (25 or 50 µg/ml) with cisplatin (5 or 10 µg/ml) also declined the percentage survival of tumour cells at each treatment duration compared to cisplatin alone (Figure 4). Lowest percentage survival was observed after incubation of cells with cisplatin and α-tocopherol for 120 min, which failed to drop further
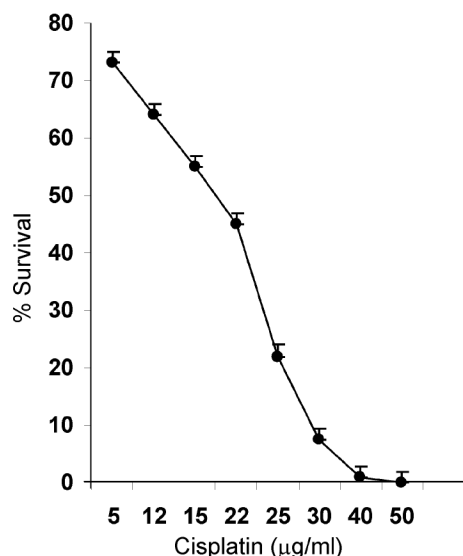

Figure 1. Survival of Dalton's lymphoma cells exposed to increasing concentrations of cisplatin for a fixed treatment duration of 1 h.

when incubation time was extended beyond 120 min. Treatment with 5 µg/ml cisplatin for 120 min reduced the survival of lymphoma cells to 46.7%, but when α-tocopherol was added to the incubation medium only 6.5–10% cells were found to survive. A similar pattern of survival was observed when the dose of cisplatin was enhanced to 10 µg/ml. Lowest surviving population was found to be 25.4% after incubation with 10 µg/ml cisplatin, which decreased to 1.5–3.4% when α-tocopherol was added to incubation medium (Figure 4). The percentage increase in cytotoxicity of cisplatin was also determined as shown in Table 1. Ten µg/ml cisplatin alone was found to be 13% more cytotoxic compared to 5 µg/ml when the treatment duration was 60 min, which improved to 27% and 46% when incubation time was

prolonged to 90 and 120 min respectively. A lower concentration of cisplatin (5 µg/ml) in combination with α-tocopherol was found to be more cytotoxic than the higher concentration of cisplatin (10 µg/ml) alone. α-Tocopherol (25 µg/ml) enhanced the cytotoxicity of 5 µg/ml cisplatin by 86%. Similarly, the higher concentration (10 µg/ml) was found to be 94% more cytotoxic when used in combination with α-tocopherol (50 µg/ml). Thus two factors responsible for enhancement of cytotoxicity of cisplatin *in vitro* are: (i) concentration of cisplatin and treatment duration, and (ii) presence of tocopherol in the medium. Due to this significant enhancement of the cytotoxic index of cisplatin, combination therapy with cisplatin + α-tocopherol was more effective than cisplatin alone[20].

The relation between DNA platination (DNA damage) and survival of lymphoma cells was also investigated. Table 2 shows platinum binding to DNA after 2 h of exposure to different concentrations of cisplatin and/or α-tocopherol, and its removal during incubation without drug(s). A direct correlation exists between the amount of platinum in the medium and DNA platination. The latter was significantly higher when the cells were incubated simultaneously with cisplatin and α-tocopherol compared to corresponding concentration of cisplatin alone. The extent of cytotoxicity may be due to decreased repair of drug-induced DNA damage. This was assessed in the present study in terms of percentage removal of platinum from DNA in a time interval of 24 h. At 24 h after the initial
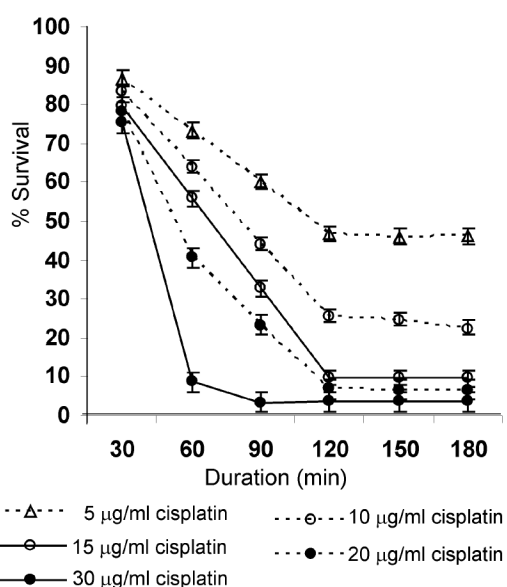

Figure 2. Effect of treatment duration on percentage survival of Dalton's lymphoma cells *in vitro*.
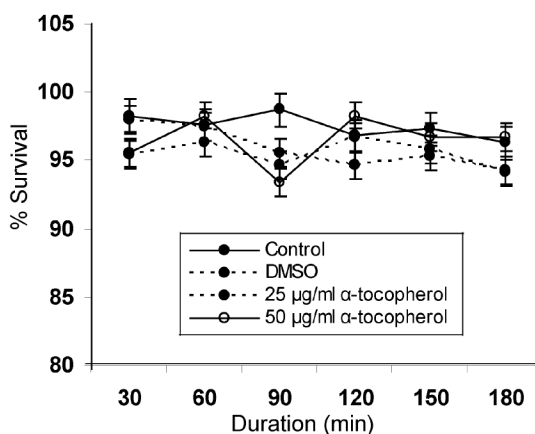

Figure 3. Survival of Dalton's lymphoma cells after treatment with α-tocopherol *in vitro* for different time durations.
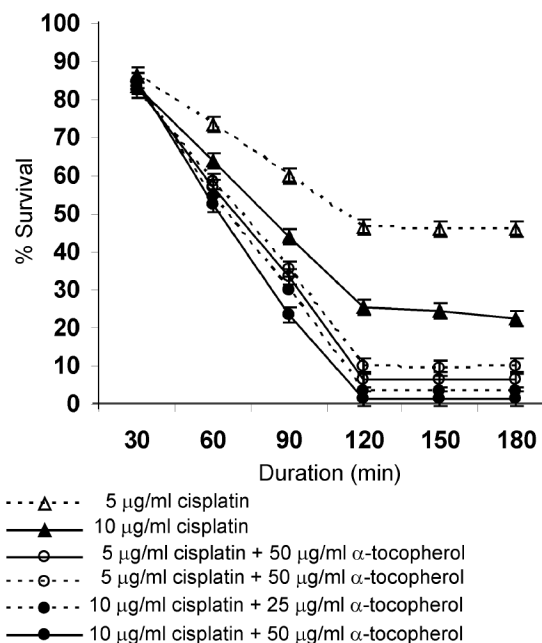

Figure 4. Survival of Dalton's lymphoma cells after treatment with cisplatin or cisplatin + α-tocopherol *in vitro* for different time durations.

Table 1. Evaluation of efficacy of combination treatment of Dalton's lymphoma in mice

Treatment	Percentage increase in cytotoxicity of cisplatin Treatment duration in min		
	60	90	120
5 µg/ml cisplatin	24*	39*	51*
10 µg/ml cisplatin	13**	27**	46**
5 µg/ml cisplatin + 25 µg/ml α-tocopherol	22**	45**	86**
5 µg/ml cisplatin + 50 µg/ml α-tocopherol	20**	41**	79**
10 µg/ml cisplatin + 25 µg/ml α-tocopherol	14***	32***	87***
10 µg/ml cisplatin + 50 µg/ml α-tocopherol	18***	47***	94***

% Increase in cytotoxicity of cisplatin = $(1 - T/C) \times 100$, where T is the survival of Dalton's lymphoma cells in the experimental system and C the survival of Dalton's lymphoma cells in control system.
*vs control; **vs 5 µg/ml cisplatin; ***vs 10 µg/ml cisplatin.

Table 2. Correlation between DNA damage (platinum content in DNA), DNA repair (percentage removal of DNA) and survival of Dalton's lymphoma cells *in vitro*

Treatment	Platinum content (pg/µg DNA)[a]		Percentage removal of DNA[d]	Percentage survival of Dalton's lymphoma cells
	0 h[b]	24 h[c]		
5 µg/ml cisplatin	45.1 ± 3.2	22.3 ± 0.7	50.5	46.7 ± 6.4
10 µg/ml cisplatin	80.2 ± 2.8*	58.3 ± 1.3*	27.3	25.4 ± 2.8
15 µg/ml cisplatin	94.8 ± 7.6*	80.7 ± 4.2*	14.9	9.8 ± 0.3
5 µg/ml cisplatin + 25 µg/ml α-tocopherol	107.3 ± 3.8*	99.6 ± 5.3*	7.2	6.5 ± 3.2
5 µg/ml cisplatin + 50 µg/ml α-tocopherol	99.3 ± 6.5*	86.2 ± 6.7*	13.2	10.0 ± 0.2
10 µg/ml cisplatin + 25 µg/ml α-tocopherol	110.3 ± 5.8**	103.2 ± 5.8**	6.4	3.4 ± 0.1
10 µg/ml cisplatin + 50 µg/ml α-tocopherol	112.4 ± 4.8**	106.7 ± 7.3**	5.0	1.5 ± 0.08

Stationary phase cultures were exposed to cisplatin or different combinations of cisplatin + α-tocopherol for 120 min.
[a]Amount of platinum bound to DNA; [b]Amount of platinum bound to DNA immediately after drug(s) removal; [c]Amount of platinum bound to DNA after 24 h of drug(s) removal; [d]Relative to the amount initially bound (0 h = 100%).
*$P < 0.001$ vs 5 µg/ml cisplatin; **$P < 0.001$ vs 10 µg/ml cisplatin.

binding (0 h), higher level of platinum remained bound to DNA in different combination groups. Each parenthesis in Table 2 shows the level of residual Pt-DNA. At 24 h, tumour cells treated with a combination of 5 µg/ml cisplatin with 25 or 50 µg/ml α-tocopherol lost 7.2% and 13.2% of their total Pt-DNA compared to 50% from cells treated with 5 µg/ml cisplatin alone. Similarly, a combination of 10 µg/ml cisplatin with 25 or 50 µg/ml α-tocopherol lost 6% and 5% of the total Pt-DNA compared to 27% from cells treated with 10 µg/ml cisplatin alone. There is a significant correlation between decrease in cell survival and the amount of platinum binding to DNA in tumour cells. More binding of Pt to DNA decreases the survival of the tumour cell (Table 2).

DNA has been reported to be the critical site for cisplatin induced cytotoxicity[21,22]. The mechanism of action of cisplatin is its ability to form covalent adducts with genomic DNA[8,23,24]. Since significantly higher intracellular platinum content was observed in the tumour cells after combination therapy with cisplatin and α-tocopherol *in vivo*[20], an attempt was made to find out whether there exists a correlation between the percentage survival of Dalton's lymphoma cells and platinum-DNA adduct formation (DNA-platination, DNA damage). It was found that decreased survival of lymphoma cells *in vitro* has a significant correlation with DNA platination. Cisplatin in combination with α-tocopherol was found to be more cyto-

toxic than the corresponding concentration of cisplatin alone and was mainly due to increased DNA platination (Table 2). Enhancement of cytotoxicity by α-tocopherol can be explained on the basis of the above results. α-Tocopherol enhances the influx of cisplatin into tumour cells, leading to increased DNA platination. Enhanced uptake of platinum by tumour cells might be due to modulation of permeability of the tumour cell membrane by altering the level of lipid peroxidation by α-tocopherol[16]. α-Tocopherol reach intra nuclear sites[17] and appeared to be associated with nucleoprotein complex which have high affinity for DNA[25]. Influx of some other anticancer agents has been reported to be enhanced by vitamin A[26,27]. Increased DNA platination by hyperthermia also enhances cytotoxicity of cisplatin-sensitive and resistant-human ovarian cell lines[28].

It was reported earlier that cytotoxicity of cisplatin is dependent upon the decreased repair of drug-induced DNA damage[14,29,30]. Table 2 shows an inverse linear correlation between DNA damage and DNA repair. Maximum DNA damage and minimum DNA repair were observed in lymphoma cells treated with 10 µg/ml cisplatin in combination with 50 µg/ml α-tocopherol, whereas the reverse was true for the group treated with 5 µg/ml cisplatin alone. Due to this relation between DNA damage and DNA repair, minimum and maximum survival of tumour cells was observed for these two groups respectively.

From the results of these studies it can be concluded that DNA repair ability would subtly be influenced by the magnitude of DNA damage. Greater damage to DNA causes an exhaustion of repair activity. The possible mechanisms of increased DNA platination by α-tocopherol could either result from increased platinum accumulation in cells or due to increased chemical reactivity of platinum in the present experimental system. This may increase retention time to withstand the high platinum-bound DNA. The platinum bound to DNA measured in the present study included both Pt-DNA mono adducts and Pt-DNA crossslinks. However, Pt-DNA assay would provide a value similar to that of crosslinks as far as the investigation of a correlation between DNA damage and cytotoxicity is concerned, since mono adducts convert to crosslinks in time[7]. In summary, α-tocopherol enhances the cytotoxicity of cisplatin in vitro and enhanced cytotoxicity is related to increased binding of platinum to DNA, which might decrease DNA repair.

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