

# $\alpha$ -Tocopherol enhances tumour growth inhibition by cis-dichlorodiammine platinum (II)

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## Abstract

Present studies indicate that  $\alpha$ -tocopherol enhances the efficacy of cisplatin as demonstrated by inoculation of Dalton's lymphoma cells incubated with either cisplatin (5 or 10  $\mu$ g/ml) alone or cisplatin +  $\alpha$ -tocopherol (25 or 50  $\mu$ g/ml) into C3H/He mice. Tumour cells ( $3 \times 10^6$  cells/mouse) incubated with cisplatin grow slowly in syngeneic mice as indicated by the late appearance of tumour. However, mice failed to develop tumour when inoculated with tumour cells incubated with cisplatin +  $\alpha$ -tocopherol. When the animals were challenged with tumour cells ( $3 \times 10^6$  cells/mouse) on the 15th day after the initial inoculation, 30-50% survived more than 60 days, with 10% tumour-free survivors being observed in some groups. Antitumour activity was higher in mice receiving lymphoma cells ( $3 \times 10^6$  cells/mouse) preincubated with cisplatin +  $\alpha$ -tocopherol compared to cisplatin alone. Tumour-bearing mice receiving cisplatin in combination with different concentrations of  $\alpha$ -tocopherol exhibited significantly higher ( $P < 0.001$ ) intratumour platinum content (123-306%) but without any change in the kidney platinum content as compared to those receiving cisplatin (5 or 10  $\mu$ g/ml) alone. Enhancement of cisplatin-induced tumour growth inhibition is probably due to the modulation of tumour cell membrane permeability by  $\alpha$ -tocopherol.  $\alpha$ -Tocopherol might increase the influx of cisplatin into tumour cells, causing the DNA repair machinery to be less efficient due to increased efficiency of adduct formation in the DNA molecule. This effect of  $\alpha$ -tocopherol can render cisplatin more effective as an antitumour agent.

## Key words

- Cisplatin
- Vitamin E
- Dalton's lymphoma
- Tumour growth inhibition

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S. Sarna is the recipient of financial assistance in the form of a research associateship from the University Grants Commission, New Delhi, India.

Received October 27, 1998  
Accepted April 10, 2000

## Introduction

Cisplatin has been used as an immunotherapeutic agent against tumours and has been reported to affect tumour cell antigenicity *in vitro* (1-4). Various cytotoxic agents have been used in combination with cisplatin to enhance its antitumour effect (5-10). Like other different combinations, thermochemotherapy with cisplatin or carboplatin has also been tried and both combinations were reported to be superior to treatment with hyperthermia alone (11). Preheating of cells

also enhanced the cytotoxic effect of cisplatin administered shortly after heating (12). The cytotoxic effect of cisplatin combined with liposomal valinomycin on human ovarian tumour cells has been reported (13). Studies on the pharmacological effect of cisplatin combined with peplomycin have shown a strong block of the G<sub>2</sub>M phase of the cell cycle resulting in the most effective killing of tumour cells (14). Clinical trials with this combination therapy have proved its usefulness for improving the clinical condition of the patients (14).

In addition to acting as an antitumour agent,  $\alpha$ -tocopherol has been reported to be effective in the inhibition of spontaneous, X-ray and chemically induced transformation of tumour cells (15-17) and to induce morphological differentiation and growth inhibition of murine and human neuroblastoma cells in culture (18).  $\alpha$ -Tocopherol has also been reported to induce apoptosis in erythroid leukemia and in prostate and breast cancer cells (19). An additive or synergistic effect of  $\alpha$ -tocopherol with a few antitumour agents has been reported for murine neuroblastoma cells both *in vitro* (20) and *in vivo* (21). Similarly, some cytotoxic chemotherapeutic agents have also been used in combination with cisplatin in an attempt to achieve enhanced antitumour effects against cisplatin-resistant tumours (7,22). Some success has been achieved but many tumour types are poorly responsive to therapy with cisplatin alone or in combination with other chemotherapeutic agents (23). Hence a lymphoma was chosen to study the synergistic effect of cisplatin and  $\alpha$ -tocopherol. In the present investigation, we studied the tumour growth pattern of Dalton's lymphoma cells *in vivo* after incubation *in vitro* with different combinations of cisplatin and  $\alpha$ -tocopherol to determine whether  $\alpha$ -tocopherol enhances the antitumour effect of a low dose of cisplatin.

### Material and Methods

Male and female mice of the C3H/He strain aged 8-10 weeks and weighing 20-22 g were used in all experiments. Mice received a commercial feed (Goldmohar, Lipton, India) and water *ad libitum*. Animals were kept under standard conditions (in a pathogen-free environment at 22-23°C, with 65-70% relative humidity).

Dalton's lymphoma was obtained from the Chittaranjan Cancer Research Center, Calcutta, India, and maintained in the laboratory by regular serial transplantations in

inbred C3H/He mice after a regular interval of 12 days.

Cisplatin was a generous gift from Bristol Myers Co., Syracuse, NY, USA. Dulbecco's modified Eagle's medium (DMEM) and glutamine were purchased from Hi-Media, Mumbai, India. Fetal bovine serum (FBS),  $\alpha$ -tocopherol, penicillin and streptomycin were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were purchased locally and were of analytical grade or equivalent.

Dalton's lymphoma cells were grown in DMEM supplemented with 10% FBS, 0.3% glutamine, 10 mM NaHCO<sub>3</sub>, and 1% penicillin (50 units/ml)-streptomycin (50 µg/ml) on culture dishes in a humidified atmosphere of 5% CO<sub>2</sub> at 37 ± 1°C. The pH was maintained at 7.2-7.4 by regulating the flow of CO<sub>2</sub>-air.

Asynchronized lymphoma cells were treated *in vitro* with cisplatin and  $\alpha$ -tocopherol before being injected into different groups of mice. Tumour cells were brought to a concentration of 3 x 10<sup>6</sup> cells/ml in DMEM containing 10% FBS, antibiotics and sodium bicarbonate. Tumour cell suspensions were incubated for 1 h at 37°C with different concentrations of cisplatin alone,  $\alpha$ -tocopherol alone, or a combination of both. The following groups were used: 1) control, 2) DMSO, 3) 25 µg/ml  $\alpha$ -tocopherol, 4) 50 µg/ml  $\alpha$ -tocopherol, 5) 5 µg/ml cisplatin, 6) 10 µg/ml cisplatin, 7) 5 µg/ml cisplatin + 25 µg/ml  $\alpha$ -tocopherol, 8) 5 µg/ml cisplatin + 50 µg/ml  $\alpha$ -tocopherol, 9) 10 µg/ml cisplatin + 25 µg/ml  $\alpha$ -tocopherol, and 10) 10 µg/ml cisplatin + 50 µg/ml  $\alpha$ -tocopherol. Viability of tumour cells was determined by the Trypan blue exclusion test. Tumour cell suspensions always exhibited more than 90% viability before incubation with any compound. Tumour cells incubated with different doses of cisplatin exhibited more than 80% viable cells, whereas in the case of  $\alpha$ -tocopherol, viability was found to be 80-90%. After incubation, 3 x 10<sup>6</sup> viable cells were injected

*ip* into different groups of mice. Groups of mice in which the tumour did not appear were challenged with tumour cells ( $3 \times 10^6$  cells/mouse) on day 15th after the initial inoculation. Increased survival time, percent survivors on different days and percent of tumour-free survivors were determined in control and experimental groups. Percent increase in the life span (%ILS) of the experimental groups was calculated using the following formula:  $\%ILS = [(T - C)/C] \times 100$ , where T = mean survival days of treated mice and C = mean survival days of untreated control mice.

For estimation of platinum content in tumour and kidney, freshly prepared cisplatin or cisplatin +  $\alpha$ -tocopherol solutions were injected *ip* into tumour-bearing mice. After 24 h mice were sacrificed, lymphoma cells were collected, counted with an electronic cell counter and washed with DMEM twice. The kidneys were removed at the same time and washed with DMEM twice. Tissues were digested with hot nitric acid to remove most of the organic matter and diluted 10-fold in 1% HCl to keep the concentration of salts below 0.2%. Platinum content was estimated with the VG plasmasquad PQ1 ICPMS apparatus. The analytical procedure completely disrupted the platinum molecule and no information was obtained about the chemical form of platinum in tissues.

Each experimental and control group consisted of 10 mice. Each set of experiment was repeated thrice and the results are reported as arithmetic mean  $\pm$  SD. Statistical significance was analyzed by the Student *t*-test.

## Results

The solvent (DMSO) failed to show any effect on tumour growth inhibition since the survival time of the control animals was not significantly different from that of animals inoculated with tumour cells treated with DMSO alone. All animals inoculated with

Dalton's lymphoma cells incubated with different concentrations of  $\alpha$ -tocopherol developed tumours. As compared to control, no significant difference in the mean survival time of mice was observed in the group inoculated with tumour cells incubated with 25  $\mu$ g/ml  $\alpha$ -tocopherol (Table 1). However, when the concentration of  $\alpha$ -tocopherol was increased to 50  $\mu$ g/ml, 10% of the animals exhibited an increase in mean survival time of 20 days. The %ILS with 25 and 50  $\mu$ g/ml  $\alpha$ -tocopherol was 7 and 17, respectively (Table 1).

Mice receiving tumour cells incubated with cisplatin (5 or 10  $\mu$ g/ml) exhibited a significant increase in mean survival time compared to control (Table 1). High concentration (10  $\mu$ g/ml) resulted in 75% 60-day survivors with a 228% increase in life span without any tumour-free survivor. However, with 5  $\mu$ g/ml, the increase in life span and 60-day survivors decreased to 141 and 17%, respectively. Animals initially inoculated with tumour cells incubated with 10  $\mu$ g/ml cisplatin and subsequently challenged exhibited a 142% increase in life span with 12% tumour-free survivors (Table 2).

Mice failed to develop tumours when inoculated with tumour cells incubated with 5 or 10  $\mu$ g/ml cisplatin along with different

Table 1 - Increased mean survival time and percent increased life span after inoculating tumour cells incubated with cisplatin and/or  $\alpha$ -tocopherol.

<sup>a</sup>Including 60-day survivors; <sup>b</sup> $[(T - C)/C] \times 100$ , where T = mean survival days of treated mice and C = mean survival days of untreated control mice; <sup>c</sup>three groups each consisting of 10 mice; P vs control (Student *t*-test). NS, Nonsignificant.

Treatment	Mean survival time in days $\pm$ SD <sup>a</sup>	% Increased life span of tumour-bearing mice <sup>b</sup>	% 60/more than 60-day tumour-bearing survivors <sup>c</sup>	% Tumour-free survivors <sup>c</sup>	P
None	18.8 $\pm$ 2.4	-	-	-	
DMSO	19.3 $\pm$ 2.7	3	-	-	NS
$\alpha$ -Tocopherol (25 $\mu$ g/ml)	20.2 $\pm$ 1.9	7	-	-	NS
$\alpha$ -Tocopherol (50 $\mu$ g/ml)	22.0 $\pm$ 3.2	17	-	-	<0.05
Cisplatin (5 $\mu$ g/ml)	45.3 $\pm$ 3.5	141	17	-	<0.001
Cisplatin (10 $\mu$ g/ml)	61.7 $\pm$ 4.8	228	75	-	<0.001

concentrations of  $\alpha$ -tocopherol. These animals when challenged on day 15th after the initial inoculation exhibited a 174% increase in life span with 30% 60-day survivors in the group receiving tumour cells incubated with 5  $\mu$ g/ml cisplatin + 25  $\mu$ g/ml  $\alpha$ -tocopherol (Table 2). The increase in the life span of the animals was found to be 170% with 40% 60-day survivors if the dose of  $\alpha$ -tocopherol was increased to 50  $\mu$ g/ml in combination with cisplatin. This combination also resulted into 10% tumour-free survivors. Similarly, the group of mice initially receiving tumour cells incubated with 10  $\mu$ g/ml cisplatin + 25  $\mu$ g/ml  $\alpha$ -tocopherol when challenged on the same day resulted into a 181% increase in life span with 38% 60-day survi-

vors. The increase in life span was found to be 203% including 50% 60-day survivors when the concentration of  $\alpha$ -tocopherol was increased to 50  $\mu$ g/ml in combination with cisplatin (Table 2).

The effect of  $\alpha$ -tocopherol on *in vivo* incorporation of cisplatin into tumour and kidney is shown in Table 3. There was a direct correlation between the concentration of cisplatin administered and platinum content in tumour and kidney.  $\alpha$ -Tocopherol significantly enhanced the intratumour platinum content. When cisplatin was combined with 20 or 40 mg/kg  $\alpha$ -tocopherol, a 256 and 306% increase in the intratumour platinum content was recorded, respectively. However, when the concentration of  $\alpha$ -tocopherol

Table 2 - Increased mean survival time, percent increase in the life span and tumour-free survivors after challenge in different groups immunized with cisplatin or cisplatin +  $\alpha$ -tocopherol-incubated tumour cells.

<sup>a</sup>Including 60-day survivors; <sup>b</sup> $[(T - C)/C] \times 100$ , where T = mean survival days of treated mice and C = mean survival days of untreated control mice; <sup>c</sup>three groups each consisting of 10 mice; P vs control (Student t-test).

Treatment	Mean survival time $\pm$ SD <sup>a</sup>	% Increased life span of tumour-bearing mice <sup>b</sup>	% 60/more than 60-day tumour-bearing survivors <sup>c</sup>	% Tumour-free survivors <sup>c</sup>	P
None	18.8 $\pm$ 2.4	-	-	-	
Cisplatin (10 $\mu$ g/ml)	45.6 $\pm$ 4.2	143	-	12.5	<0.001
Cisplatin (5 $\mu$ g/ml) + $\alpha$ -tocopherol (25 $\mu$ g/ml)	51.5 $\pm$ 5.8	174	30	-	<0.001
Cisplatin (5 $\mu$ g/ml) + $\alpha$ -tocopherol (50 $\mu$ g/ml)	50.8 $\pm$ 6.3	170	40	10	<0.001
Cisplatin (10 $\mu$ g/ml) + $\alpha$ -tocopherol (25 $\mu$ g/ml)	52.4 $\pm$ 5.7	181	38	-	<0.001
Cisplatin (10 $\mu$ g/ml) + $\alpha$ -tocopherol (50 $\mu$ g/ml)	57.0 $\pm$ 7.4	203	50	-	<0.001

Table 3 - Platinum content in kidney and tumour after treatment with cisplatin alone or in combination with  $\alpha$ -tocopherol.

\*P<0.001 vs 3 mg/kg cisplatin (Student t-test). \*\*Nonsignificant vs 3 mg/kg cisplatin. Figures in parentheses indicate the percent increase in intratumour platinum content after treatment with cisplatin +  $\alpha$ -tocopherol.

Treatment	Platinum content ( $\mu$ g/g wet tissue)	
	Tumour	Kidney
3 mg/kg cisplatin	0.80 $\pm$ 0.05	6.28 $\pm$ 1.37
9 mg/kg cisplatin	3.82 $\pm$ 0.18* (377%)	12.98 $\pm$ 2.67*
3 mg/kg cisplatin + 20 mg/kg $\alpha$ -tocopherol	2.85 $\pm$ 0.52* (256%)	6.67 $\pm$ 1.90**
3 mg/kg cisplatin + 40 mg/kg $\alpha$ -tocopherol	3.25 $\pm$ 0.46* (306%)	6.95 $\pm$ 1.22**
3 mg/kg cisplatin + 60 mg/kg pms-tocopherol	1.79 $\pm$ 0.60* (123%)	6.65 $\pm$ 1.50**

was enhanced to 60 mg/kg, the increase in platinum content was reduced to 123%.  $\alpha$ -Tocopherol failed to show any effect on the platinum content of the kidney.

## Discussion

Vitamins are antioxidants since they prevent the toxic effects of free radicals, which can interact with a variety of cellular molecules causing inhibition or alteration of functions through lipid peroxidation, DNA damage and enzyme inactivation. Vitamins possess chemical properties that allow them to regulate the oxidation-reduction potential of the cell and are frequently used in cancer therapies (24).

The present study indicates that  $\alpha$ -tocopherol enhances the tumour growth inhibition induced by a low dose of cisplatin.  $\alpha$ -Tocopherol alone had no significant effect on tumour growth inhibition, with only 10% of the animals showing increased survival time when inoculated with tumour cells incubated with 50  $\mu$ g/ml  $\alpha$ -tocopherol. It has been reported that  $\alpha$ -tocopherol slightly enhances the proliferation (25) and inhibits the differentiation (26) of mouse myeloid leukemia cells. The growth of a variety of cell types is inhibited by vitamin E succinate (27-29) whereas d- $\alpha$ -tocopherol or its analogues such as acetate and nicotinate esters as well as the water-soluble analogue Trolox had very little effect on cell growth and differentiation *in vitro* (27). It has been suggested that vitamin E succinate, having a negative charge, membrane permeability and stability in intracellular compartments, inhibits cell growth and transformation, at least in part due to the inhibition of protein kinase C activity. However, vitamin E succinate did not significantly affect the growth of melanoma cells in culture (24).

Mice inoculated with cisplatin incubated with tumour cells exhibited a significant increase in their mean survival time compared to control. Palpable tumour was observed on

day 15 in the group receiving tumour cells incubated with 5  $\mu$ g/ml cisplatin, but when the concentration of cisplatin was enhanced to 10  $\mu$ g/ml, tumour failed to appear on the same day. Even a challenge of the latter group with tumour cells resulted into an increase in the mean survival time of mice compared to control with 12% tumour-free survivors (Table 2). This shows that cisplatin brings about some changes in lymphoma cells responsible for the late appearance of the tumour. These changes may be morphological involving loss of membrane integrity and cell shrinkage (30,31). Cell surface and biochemical alterations have been reported to occur when fibrosarcoma cells are treated *in vitro* with cisplatin (3,32). In the present system a greater therapeutic benefit was achieved with 10  $\mu$ g/ml cisplatin which resulted into increased mean survival time of animals even after the challenge. This concentration of cisplatin was shown to release a maximum amount of sialic acid from tumour cells if incubated *in vitro* (32) and may have been responsible for this effect. A synergistic effect of cisplatin and  $\alpha$ -tocopherol was also apparent in the present study. The therapeutic potential of cisplatin was greatly enhanced by  $\alpha$ -tocopherol since tumour cells incubated with various combinations of cisplatin +  $\alpha$ -tocopherol failed to grow in animals. The mean survival time of animals increased even after challenge in different combination groups. This shows that tumour cells are viable after 1 h of drug treatment, being able to enhance the immune response but unable to grow into a tumour. Recently, it has been reported that after treatment with cisplatin and  $\alpha$ -tocopherol these tumour cells progress towards necrosis (33). A marked inhibition of tumour growth was reported earlier when cisplatin was directly inoculated into tumour-bearing mice in combination with vitamins (24,34).

Therapeutic effect of different doses of cisplatin is directly related to the intratumour platinum content. In the present study, a

subtherapeutical dose of cisplatin (3 mg/kg) was used in combination with  $\alpha$ -tocopherol in order to elevate the intratumour platinum content as well as to observe its nephrotoxic effect. The present study suggests that one of the causes of enhancement of cisplatin-induced tumour growth inhibition by  $\alpha$ -tocopherol might increase the uptake of cisplatin into tumour cells. This process is specific because platinum content was elevated only in the tumour and not in the kidney of the animals receiving  $\alpha$ -tocopherol along with cisplatin. The increased uptake of cisplatin by  $\alpha$ -tocopherol is suggested to be the result of the modulation of cell membrane permeability altering the level of peroxidation (18). In addition, membrane electropermeabilisation also affects both influx and efflux of cisplatin from tumour cells (35). The influx of some anticancer agents has been reported to be increased by vitamin A, which inhibits the growth of neuroblastoma cells (36-39). In addition to the effect of cisplatin on cell permeability, DNA is the critical target for cisplatin-induced cytotoxicity (40). The mechanism involved in the action of cisplatin is its ability to form covalent adducts with the genomic molecules (41-44). This process renders the cells incapable of replication, with consequent cell death (40). Cisplatin has been reported to induce programmed cell death or apoptosis that is triggered by the presence of cisplatin-DNA adducts; however, the signal transduction mechanism that links DNA damage to the cell death pathway remains unknown (30).  $\alpha$ -Tocopherol may also act at the site of DNA and RNA where cisplatin binds (45), possibly increasing the efficiency of adduct for-

mation in the genome, thus decreasing the ability of cells to remove these adducts. Such a decrease in the efficiency of the DNA repair machinery of tumour cells enables cisplatin to be more effective as an antitumour agent. Another possibility is that vitamins exert antineoplastic effects by increasing cytolytic and autophagic activity, cell membrane disruption and increased collagen synthesis, thus inhibiting the cancer cell metabolism and proliferation (46) or by stimulating antitumour immunity. Stimulation of helper T cells might enhance the antitumour activity of cisplatin (21).  $\alpha$ -Tocopherol has been reported to enhance both lymphoproliferative reactions and the antitumour effect of adriamycin (47,48).

The present results indicate that combined treatment of Dalton's lymphoma with cisplatin and  $\alpha$ -tocopherol has some advantage over cisplatin alone. The enhancement of tumour growth inhibition by  $\alpha$ -tocopherol is possibly due to its effects on the influx of cisplatin into tumour cells, increasing the efficiency of adduct formation in genomic DNA and thus weakening the DNA repair machinery of tumour cells. This effect of  $\alpha$ -tocopherol renders cisplatin more effective as an antitumour agent and indicates the importance and function of vitamins in cancer therapy.

### Acknowledgments

We are thankful to Dr. D.T. Khating, Head, Regional Sophisticated Instrumentation Centre, Shillong, for providing the facilities for platinum measurement.

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