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ORIGINAL ARTICLE

Chemoprotection and enhancement of cancer chemotherapeutic efficacy of cyclophosphamide in mice bearing Ehrlich ascites carcinoma by diphenylmethyl selenocyanate

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Abstract
Purpose Chemoprotective effect of diphenylmethyl selenocyanate against cyclophosphamide (CP) induced cellular toxicity and antitumor efficacy was evaluated in mice bearing Ehrlich ascites carcinoma.
Methods Diphenylmethyl selenocyanate (3 mg/kg.b.w.) was administered orally and CP was given intraperitoneally (25 mg/kg.b.w). The effects were observed on the level of lipid peroxidation, antioxidant enzymes status, serum transaminase (ALT, AST) activity, hematological profile, transplantable murine tumor growth, apoptosis induction in tumor cells, and life span of tumor bearing hosts.
Results The selenium compound restored the levels of antioxidant enzymes system, decreased the level of lipid peroxidation and serum transaminase activity. Hematological profile reverted to near normal level after selenium compound treatment. Treatment with the selenium compound also resulted in significant tumor growth regression along with significant upregulation of apoptosis, increased in mean survival time and life span of tumor bearing host.
Conclusions Results clearly indicate that diphenylmethyl selenocyanate has the potential to reduce the cellular toxicity of CP at the same time improving its antitumor efficacy.

Keywords Cyclophosphamide · Diphenylmethyl selenocyanate · Oxidative stress · Antioxidant · Antitumor activity

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Introduction

Treatment of cancer by chemotherapy is often accompanied by toxic side effects caused in part by oxidative stress induced by chemotherapeutic drugs [1]. Cyclophosphamide (CP), a widely used drug in cancer chemotherapy is also not free of such complications [2]. Numerous studies reveal that CP induces oxidative stress and is cytotoxic to normal cells. Generation of reactive oxygen species (ROS) by CP results in enhanced lipid peroxidation (LPO) and reduction in tissue antioxidant enzymes system and is one of the mechanism by which CP and its metabolites exert their toxic effects in different tissues of experimental animals [3, 4].

A novel strategy to prevent chemotherapy induced toxicity based on the administration of antioxidants to reduce side effects caused by the occurrence of oxidative stress [5]. Antioxidants are natural or synthetic molecules preventing the uncontrolled formation of ROS or inhibiting their reactions with biological structures. Selenium is considered to be highly potent and a well established nutritional antioxidant for animal and human beings, this nutrient is an important part of antioxidant enzymes that protect cells against the harmful effects of free radicals [6]. There is increasing evidence that selenium can act as an anticarcinogen and inhibit tumor initiation and progression [7, 8]. However, the use of the main known dietary sources of selenium, such as selenomethionine, selenocysteine, and inorganic selenium such as sodium selenite are limited due to their toxicity [9, 10]. Therefore the development of new organoselenium compound with higher cancer chemopreventive efficacy and better tolerance continues to be a priority in cancer chemoprevention research. Attempts are being made by various laboratories including our own, to develop new organic forms of selenium compounds with reduced toxicity and enhanced cancer chemopreventive efficacy. Diphenylmethyl

selenocyanate, a synthetic organoselenium compound was reported earlier as a potential cancer chemopreventive agent against chemically induced murine toxicity and carcinogenesis models in vivo [11, 12]. The present study was carried out to evaluate the chemoprotective efficacy of diphenylmethyl selenocyanate against CP induced toxicity in mice bearing Ehrlich ascites carcinoma (EAC) cells, on the basis of changes in some enzymatic as well as non enzymatic antioxidants and hematological parameters in tumor bearing mice.

Materials and methods

Animals

Adult (5–6 weeks) Swiss albino male mice (23 ± 2 g), bred in the animal colony of Chittaranjan National Cancer Institute, Kolkata, used for this study, were maintained at controlled temperature under alternating light and dark conditions. Standard food pellets (Lipton India Ltd) and drinking water was provided ad libitum. The experiments were carried out following strictly the Institute's guideline for the care and use of laboratory animals.

Tumor cells

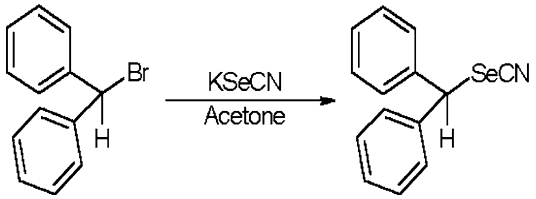
EAC cells was maintained in Swiss mice by weekly intraperitoneal (i.p.) transplantation of 1×10^6 viable tumor cells suspended in phosphate buffer saline (PBS).

Chemicals

Cyclophosphamide was obtained from Cadila Pharmaceuticals (India). Diphenylmethyl bromide, potassium selenocyanate (KSeCN), 1-Chloro-2, 4-dinitrobenzene (CDNB), ethylene diamine tetra acetic acid (EDTA), reduced glutathione (GSH), pyrogallol, 5,5′-dithio-bis(2-nitro benzoic acid)(DTNB), sodium dodecyl sulphate (SDS), bovine serum albumin (BSA), β-nicotinamide adenine dinucleotide phosphate (reduced) (β-NADPH) and glutathione reductase were obtained from Sigma-Aldrich Chemicals Private Limited, India. Hydrogen peroxide (H_2O_2) 30%, propylene glycol, hexane, and thioberbituric acid (TBA) were obtained from Merck Specialties Private Limited, India. Serum ALT and AST assay kits were obtained from Span Diagnostics Limited, India. In situ cell detection kit, AP was purchased from Roche Diagnostics India Private Limited.

Synthesis of the compound

Diphenylmethyl selenocyanate was prepared following a literature procedure [13]. Briefly, diphenylmethyl bromide was treated with KSeCN in acetone at 60–70°C for 5 h. Acetone was removed under reduced pressure and the resulting solid was extracted with diethyl ether. Usual work up then afforded the desired compound (1), which was crystallized from hexane to get a colorless crystalline solid m.p. 68–69°C.



(1)

Drug preparation

Synthetic organoselenium compound diphenylmethyl selenocyanate was used as a suspension in 5.5% propylene glycol in water.

Experimental design

Animals were divided into seven groups Gr. (I–VII) each group consisting of 12 animals. Six animals from each group were taken for the study of biochemical, hematological parameters, and antitumor activity. The rest of the animals in each of the groups were kept to check the mean survival time of animals in each group. Animals of Gr. (II–VII) were injected with EAC cells (1×10^6 cells/mouse) intraperitoneally. The day of EAC cell inoculation was count as day zero. No treatment was given on the day of EAC cell inoculation. The groups were treated as follows:

Gr. I (Vehicle control): Animals were given 5.5% propylene glycol in water by oral gavage from day 1 to day 6.

Gr. II (EAC control): Animals were given 5.5% propylene glycol in water by oral gavage from day 1 to day 6.

Gr. III (CP only treated group): Animals were given CP at a dose of 25 mg/kg.b.w. in water by intraperitoneal administration and received 5.5% propylene glycol in water by oral gavage from day 1 to day 6.

Gr. IV (Diphenylmethyl selenocyanate only treated group): Animals were pretreated with diphenylmethyl selenocyanate orally at a dose of 3 mg/kg.b.w. 7 days prior to tumor inoculation and the treatment continued 24 h after tumor inoculation from day 1 to day 6. (The day of EAC cell inoculation was count as day zero).

Gr. V (Diphenylmethyl selenocyanate only treated group): Animals were treated only with diphenylmethyl selenocyanate (3 mg/kg.b.w.) 24 h after tumor inoculation from day 1 to day 6.

Gr. VI (Diphenylmethyl selenocyanate + CP treated group): Diphenylmethyl selenocyanate (3 mg/kg.b.w.) was administered orally 7 days prior to tumor inoculation and then continued 24 h after tumor inoculation along with CP (25 mg/kg.b.w.) from day 1 to day 6.
Gr. VII (Diphenylmethyl selenocyanate + CP treated group): Diphenylmethyl selenocyanate (3 mg/kg.b.w.) along with CP (25 mg/kg.b.w.) was administered from day 1 to day 6.

Twenty-four hours after the last dose, six animals from each group were sacrificed, and the parameters described below were studied.

Biochemical estimation

Quantitative estimation of LPO

LPO was estimated in liver and lung microsomal fraction. The level of lipid peroxides formed was measured using thiobarbituric acid and expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein using extinction co-efficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [14].

Estimation of GSH level

GSH level was estimated in liver and lung cytosol spectrophotometrically by determination of DTNB reduced by –SH groups by measuring the absorbance at 412 nm. The level of GSH was expressed as nmol/mg^{-1} of protein [15].

Estimation of glutathione -S-transferase (GST) activity

GST activity in liver and lung cytosol was determined from the increase in absorbance at 340 nm with CDNB as the substrate and specific activity of the enzyme expressed as formation of CDNB–GSH conjugate/min/mg of protein [16].

Estimation of glutathione peroxidase (GPx) activity

GPx activity in liver and lung tissue sample was determined by NADPH oxidation using a coupled reaction system consisting of reduced glutathione, glutathione reductase, and hydrogen peroxide [17]. Briefly, 100 µl of enzyme sample was incubated for 10 min with 800 µl reaction mixture (0.25 M potassium phosphate buffer containing 2.5 mM EDTA and 2.5 mM sodium azide, 10 mM reduced glutathione, 2.5 mM NADPH, and 2.4 units of glutathione reductase). The reactions started by adding 100 µl H_2O_2 and follow the decrease in NADPH absorbance at 340 nm for 3 min. The enzyme activity was expressed as µmol NADPH utilised/min/mg of protein using extinction coefficient of NADPH at 340 nm as 6,200 $M^{-1}\ cm^{-1}$.

Estimation of catalase (CAT) activity

Activity of CAT in liver and lung tissue sample was determined spectrophotometrically at 250 nm and expressed as unit/mg of protein where the unit is the amount of enzyme that liberates half the peroxide oxygen from H_2O_2 in seconds at 25°C [18].

Estimation of Superoxide dismutase (SOD) activity

SOD activity in liver and lung tissue sample was determined by quantification of pyrogallol auto oxidation inhibition and the amount of enzyme necessary for inhibiting the reaction by 50%. Auto oxidation of pyrogallol in Tris–HCl buffer (50 mM, pH 7.5) is measured by increase in absorbance at 420 nm [19, 20].

Determination of serum alanine transaminase (ALT) and aspartate transaminase (AST) activity

Serum were collected from blood sample of mice by centrifugation, then the serum ALT, AST levels were measured spectrophotometrically by standard enzymatic method using commercial kits.

Hematological studies

Hemoglobin (Hb) content of blood samples was measured following Sahli's method [21]. Red blood cell (RBC) and white blood cell (WBC) counts were made following a literature procedure [22, 23]. Differential WBC count [24] was carried out from Leishaman stained blood smears.

Estimation of protein

Protein was estimated spectrophotometrically [25] with bovine serum albumin as standard.

Tumor growth response

The antitumor effect of CP along with diphenylmethyl selenocyanate was assessed by measuring the changes in ascites tumor volume, packed cell volume and viable tumor cell count. Mean survival time (MST) of each group containing six mice was monitored and percentage increase in life span (% ILS) was calculated using following equation [26, 27]. MST = (Day of first death + Day of last death)/2. ILS (%) = [(Mean survival time of treated group/mean survival time of control group) − 1] × 100.

In situ cell death detection (apoptosis)

Apoptosis of EAC cells were determined by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end labelling (TUNEL) method with the help of in situ cell death detection kit, AP [28].

Statistical analysis

The differences in mean values of different groups were tested and the values are expressed as mean ± SD. The data were analyzed using the student's t test and $P < 0.05$ was considered to be significant.

Results

Microsomal LPO level

In order to determine the oxidative damage triggered by cyclophosphamide and the effect of diphenylmethyl selenocyanate on this event, the degree of lipid peroxidation was examined. The level of LPO in liver and lung tissues were increased significantly ($P < 0.05$) by 30.3 and 52.3% in the EAC control group (Gr. II) as compared to the vehicle control (Gr. I) (Fig. 1a, b). CP treatment of tumor bearing mice for 6 days showed a further significant increase of LPO level in liver and lung by 23.2 and 22.2% (Gr. III) in comparison to the EAC control. The compound diphenylmethyl selenocyanate itself reduced LPO level by 30.3 (Gr. IV) and 24.2% (Gr.V) in liver and by 47.6 (Gr. IV) and 40.4% (Gr. V) in lung as compared with EAC control. Diphenylmethyl selenocyanate during CP treatment also reduced the level of LPO in liver and lung tissue significantly ($P < 0.05$) by 41.8 and 55.5% in case of 7 days pretreatment (Gr. VI) and by 32.5 and 46.2% in case of concomitant treatment (Gr. VII) as in comparison to CP only treated group.

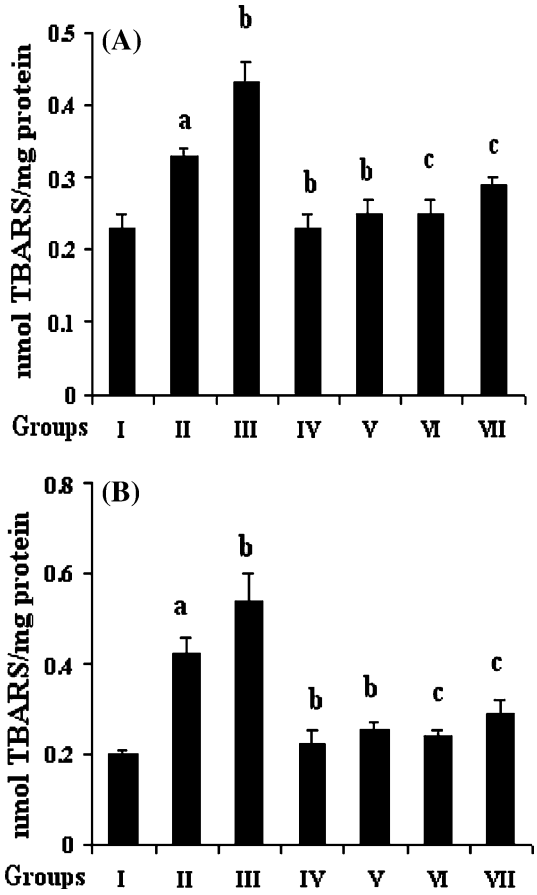


Fig. 1 Effect of diphenylmethyl selenocyanate on LPO level of CP treated EAC bearing mice liver (**A**) and lung (**B**). Values are expressed as mean ± SD, $n = 6$. [a]$P < 0.05$ statistically compared with Gr. I (Vehicle control), [b]$P < 0.05$ statistically compared with Gr. II (EAC control), [c]$P < 0.05$ statistically compared with Gr. III (CP only treated group)

Esitmation of nonenzymatic and enzymatic antioxidants

In order to investigate whether the antioxidant properties of diphenylmethyl selenocyanate were mediated by an increase in nonenzymatic and enzymatic antioxidants, GSH,GST, GPX, CAT, and SOD activities were measured.

GSH level

Inoculation of EAC drastically decrease the GSH content in liver and lung by 54.4 and 35.9% in the EAC control (Gr. II) when compared with the vehicle control (Gr. I) (Fig. 2a, b). CP treatment of tumor bearing mice showed a further significant decrease of GSH level in liver by 37.05% and in lung by 41.6% in comparison to Gr. II. Diphenylmethyl selenocyanate itself increased GSH level in liver by 56.3 (Gr. IV) and 50.6% (Gr. V) and in lung by 57.5 (Gr. IV) and 48.5% (Gr. V) as compared with Gr. II. Diphenylmethyl selenocyanate during CP treatment also significantly ($P < 0.05$) increased the GSH level in liver and lung by 69.6 and 70.01% in case of 7 days pretreatment (Gr. VI), and by 57.4 and 56.1% in case of concomitant treatment (Gr. VII) as in comparison to CP only treated group(Gr. III).

GST activity

The activity of GST in the liver and lung of EAC bearing mice decreased significantly ($P < 0.05$) by 38.0 and 33.2% as compared with vehicle control (Fig. 3a, b). After intraperitoneal administration of CP, GST activity in liver and lung further decreased by 36.3 and 25.3% in comparison to the EAC control group (Gr. II). Diphenylmethyl selenocyanate

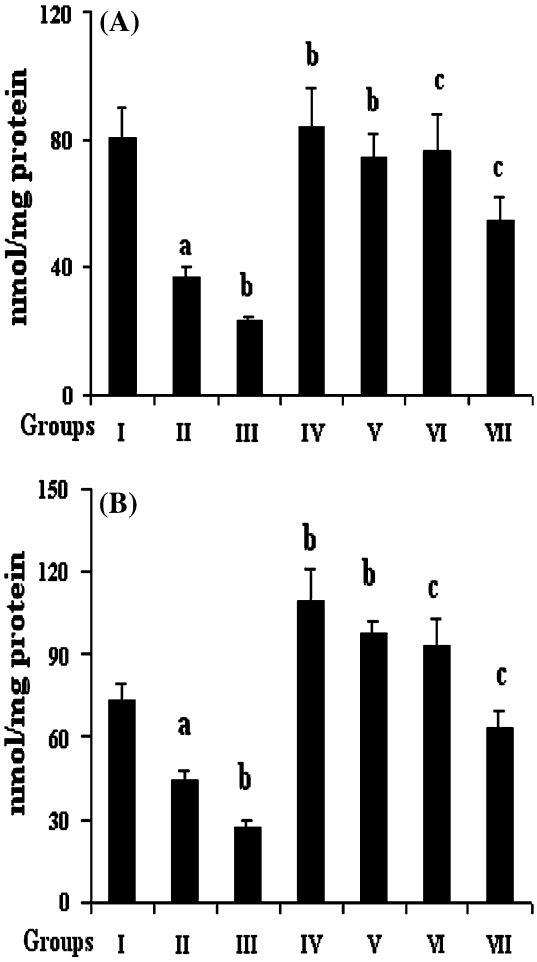


Fig. 2 Effect of diphenylmethyl selenocyanate on GSH level of CP treated EAC bearing mice liver (**A**) and lung (**B**). Values are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$ statistically compared with Gr. I (Vehicle control), $^{b}P < 0.05$ statistically compared with Gr. II (EAC control), $^{c}P < 0.05$ statistically compared with Gr. III (CP only treated group)

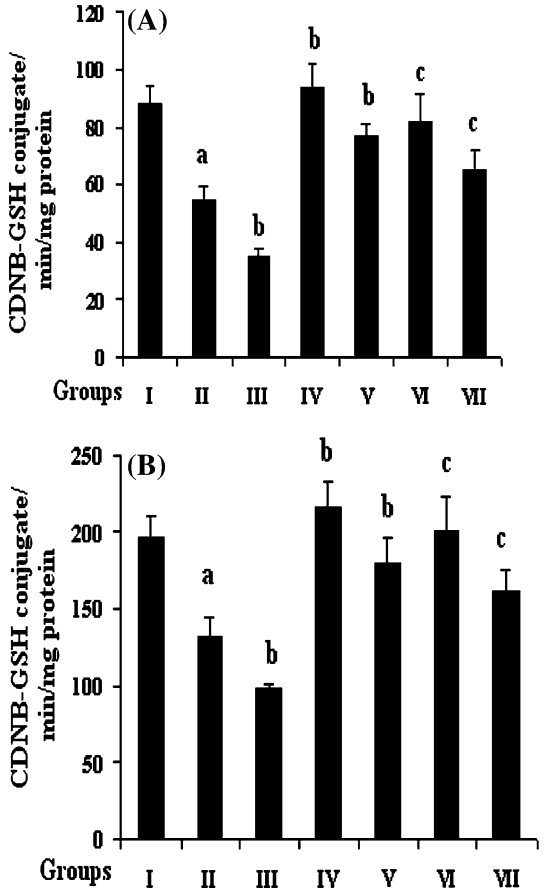


Fig. 3 Effect of diphenylmethyl selenocyanate on GST activity of CP treated EAC bearing mice liver (**A**) and lung (**B**). Values are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$ statistically compared with Gr. I (Vehicle control), $^{b}P < 0.05$ statistically compared with Gr. II (EAC control), $^{c}P < 0.05$ statistically compared with Gr. III (CP only treated group)

itself increased GST activity in liver by 41.7 (Gr. IV) and 29.2% (Gr. V) and in lung by 38.9 (Gr. IV) and 27.2% (Gr. V) as compared with Gr. II. Diphenylmethyl selenocyanate also sharply increased GST activity during CP treatment in liver and lung by 57.5 and 51.2% in case of 7 days pretreatment (Gr. VI), however concomitant treatment (Gr. VII) with the same compound elevated GST activity by 46.9 and 39.2% as compared to CP only treated group.

GPx activity

GPx activity in liver and lung of EAC bearing mice decreased significantly ($P < 0.05$) by 43.18 and 66.1% as compared with vehicle control (Gr. I) (Fig. 4a, b). After intraperitoneal administration of CP, GPx activity in liver and lung further decreased by 34 and 58.5% in comparison to the EAC control (Gr. II). Diphenylmethyl selenocyanate itself increased GPx activity in liver by 39.0 (Gr. IV) and 19.35% (Gr. V) and in lung by 67.2 (Gr. IV) and 56.5% (Gr. V) as compared with Gr. II. Diphenylmethyl selenocyanate also increased GPx activity during CP treatment in liver and lung by 53.5 and 86.1% respectively in case of pretreatment (Gr. VI), and by 44.0 and 79.2% respectively in case of concomitant treatment (Gr. VII) as compared to CP only treated group.

CAT activity

CAT activity in the liver of EAC control group significantly decrease by 43.3% in comparison with the vehicle control (Gr. I) (Fig. 5a). CP treatment further decreased CAT activity significantly by 37.5% in comparison with the EAC control group. The compound diphenylmethyl selenocyanate alone were able to increased CAT activity in liver by 40.1 (Gr. IV) and 23.5% (Gr. V) in comparison to Gr. II. Diphenylmethyl selenocyanate during CP treatment also

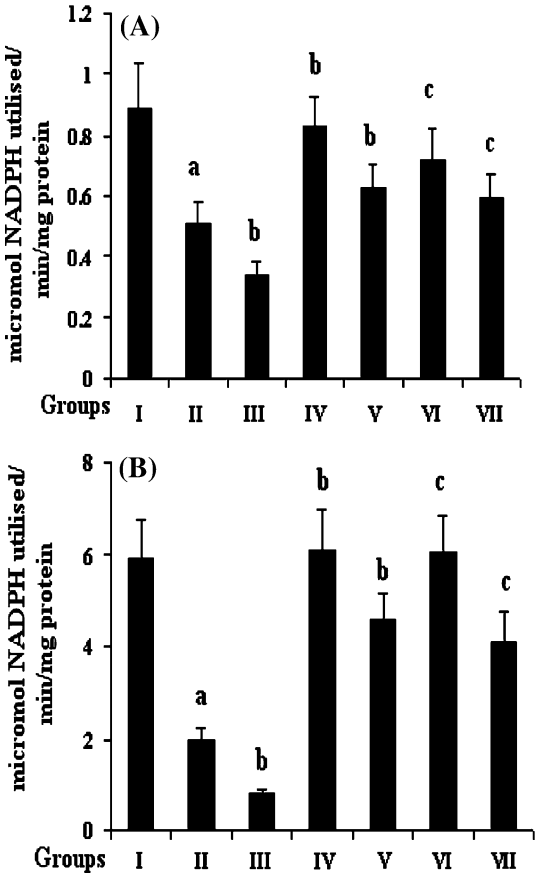


Fig. 4 Effect of diphenylmethyl selenocyanate on GPx activity of CP treated EAC bearing mice liver (**A**) and lung (**B**). Values are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$ statistically compared with Gr. I (Vehicle control), $^{b}P < 0.05$ statistically compared with Gr. II (EAC control), $^{c}P < 0.05$ statistically compared with Gr. III (CP only treated group)

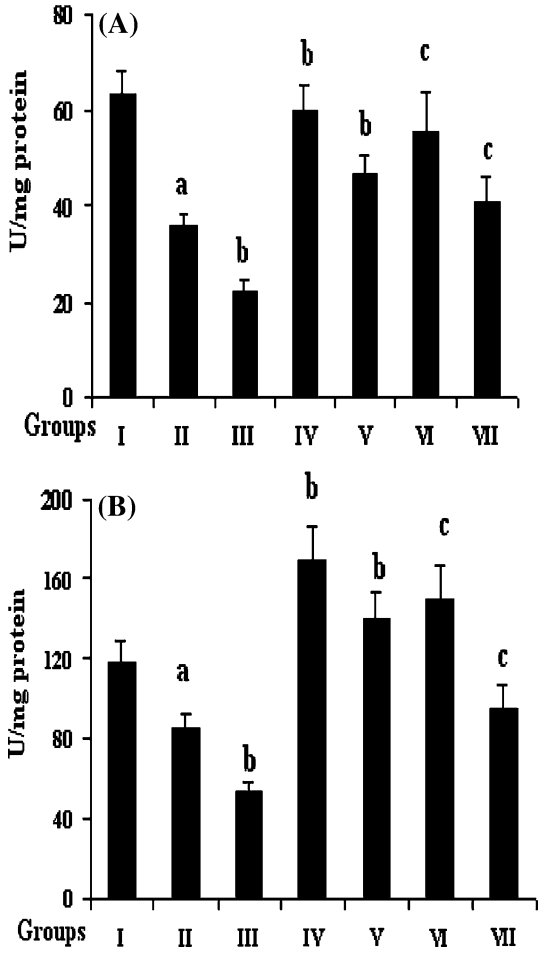


Fig. 5 Effect of diphenylmethyl selenocyanate on CAT activity in liver of CP treated EAC bearing mice (**A**). Effect of diphenylmethyl selenocyanate on SOD activity in liver of CP treated EAC bearing mice (**B**). Values are expressed as mean ± SD, $n = 6$. $^{a}P < 0.05$ statistically compared with Gr. I (Vehicle control), $^{b}P < 0.05$ statistically compared with Gr. II (EAC control), $^{c}P < 0.05$ statistically compared with Gr. III (CP only treated group)

significantly ($P < 0.05$) enhanced the enzyme activity in liver by 59.8% in case of 7 days pretreatment (Gr. VI) and by 45.4% in case of concomitant treatment (Gr. VII). No significant alteration of CAT activity was observed in the lung cytosol.

SOD activity

SOD activity in the liver of EAC bearing mice significantly decreased by 27.7% as in comparison with the vehicle control (Gr. I) (Fig. 5b). CP treatment further decreased the activity in liver by 37.01% (Gr. III) as in comparison with EAC control (Gr. II). The compound diphenylmethyl selenocyanate alone were able to increased SOD activity in liver by 49.6 (Gr. IV) and 39.04% (Gr. V) as compared to EAC control. Diphenylmethyl selenocyanate during CP treatment also significantly ($P < 0.05$) elevated the activity by 64.2% in case of 7 days pretreatment (Gr. VI) and by 43.9% in case of concomitant treatment (Gr. VII). No significant alteration of SOD activity was observed in the lung cytosol.

Effect of diphenylmethyl selenocyanate along with CP on hematological parameters and serum transaminase (ALT and AST) activity

Hematological parameters of tumor bearing mice altered significantly compared to the normal mice (Table 1). The total WBC count was found to be increased with a reduction of Hb content and RBC count. In differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased significantly ($P < 0.05$) in tumor bearing mice compared to normal mice (Gr. I). CP administration result in further decreased in WBC, RBC counts and Hb content. In differential count of WBC, the percent of neutrophils and lymphocyte count also

Table 1 Effect of diphenylmethyl selenocyanate and CP on hematological parameters and serum transaminase (ALT and AST) activity

Groups	Hb content (g/dl)	RBC (10^6/mm^3)	WBC (10^3/mm^3)	Neutrophil (%)	Lymphocyte (%)	ALT (U/ml)	AST (U/ml)
I	12.7 ± 0.3	6.1 ± 0.2	6.2 ± 0.3	9.3 ± 0.7	87 ± 8.2	11.5 ± 0.5	45.2 ± 4.5
II	9.8 ± 0.2[a]	5.0 ± 0.1[a]	16.3 ± 1.8[a]	32 ± 3.5[a]	58.6 ± 5.4[a]	21.7 ± 2.3[a]	81.7 ± 8.8[a]
III	8.3 ± 0.1[b]	4.0 ± 0.1[b]	11.4 ± 0.3[b]	20.8 ± 1.1[b]	35.2 ± 4.1[b]	40.5 ± 4.2[b]	136.6 ± 11.5[b]
IV	11.8 ± 0.2[b]	6.1 ± 0.1[b]	7.2 ± 0.1[b]	14.8 ± 1.3[b]	86.5 ± 5.7[b]	10.2 ± 1.2[b]	40.3 ± 2.5[b]
V	11.0 ± 0.5[b]	6.0 ± 0.05[b]	7.6 ± 0.9[b]	16.1 ± 1.2[b]	68 ± 5.1[b]	14.9 ± 1.0[b]	49.7 ± 3.8[b]
VI	11.6 ± 0.7[c]	6.1 ± 0.2[c]	7.1 ± 1.1[c]	14.7 ± 0.8[c]	85 ± 7.8[c]	15.3 ± 1.1[c]	45.3 ± 2.3[c]
VII	10.8 ± 0.7[c]	5.8 ± 0.3[c]	7.3 ± 1.1[c]	17.4 ± 0.6[c]	64 ± 4.8[c]	18.8 ± 2.3[c]	63.6 ± 6.3[c]

Values are expressed as mean ± SD, $n = 6$

[a] $P < 0.05$ statistically compared with Gr. I (Vehicle control)

[b] $P < 0.05$ statistically compared with Gr. II (EAC control)

[c] $P < 0.05$ statistically compared with Gr. III (CP only treated group)

decreased. Diphenylmethyl selenocyanate alone (Gr. IV, Gr. V) and during CP treatment (Gr. VI, Gr. VII) results in changed these altered parameters more or less normal. Tumor growth was associated with alteration in liver function as revealed by sharp rise in serum ALT and AST activities (Table 1). The activity of ALT in serum of EAC bearing mice increased significantly ($P < 0.05$) by 47.12% (Gr. II) as compared with vehicle control (Gr. I). After CP treatment (Gr. III) ALT activity in serum again increased by 46.29% in comparison to the EAC control group. Diphenylmethyl selenocyanate itself reduced the ALT activity by 52.8 (Gr. IV) and 31.49% (Gr. V) as in comparison to Gr. II. Diphenylmethyl selenocyanate during CP treatment also significantly reduced the ALT activity by 62.14% in case of 7 days pretreatment (Gr. VI) and by 53.4% in case of concomitant treatment (Gr. VII) as in comparison with Gr. III. There was also a significant rise in AST activity by 44.6% in the EAC control group (Gr. II) as compared to vehicle control (Gr. I) (Table 1). CP treatment again increased the AST activity by 40.1% in comparison to EAC control group (Gr. II). Diphenylmethyl selenocyanate itself decreased AST activity by 50.6 (Gr. IV) and 39.1% (Gr. V) as compared to EAC control group (Gr. II). Diphenylmethyl selenocyanate during CP treatment also decreased AST activity significantly by 66.8% in case of 7 days pretreatment (Gr. VI) and by 53.41% in case of concomitant treatment (Gr. VII) as compared to Gr. III.

Effect of diphenylmethyl selenocyanate along with CP on tumor growth, mean survival time, % ILS and apoptosis

Antitumor activity of CP along with diphenylmethyl selenocyanate against EAC bearing mice was assessed by the parameters such as tumor volume, packed cell volume, viable cell count, mean survival time, and % ILS (Table 2). It has been observed that the mean ± (SD) tumor volume and packed cell volume of control EAC bearing mice (Gr. II) at day 7 after tumor transplantation were 2.5 ± 0.3 and 1.5 ± 0.4 ml respectively. After administration of CP (Gr. III), diphenylmethyl selenocyanate (Gr. IV, Gr. V) and a combination of both CP plus diphenylmethyl selenocyanate (Gr. VI, Gr. VII) the mean ± (SD) tumor volume were 0.4 ± .05, 1.6 ± 0.2, 1.7 ± 0.08, 0.2 ± 0.1, 0.3 ± 0.05 and packed cell volume were 0.27 ± 0.09, 0.75 ± 0.2, 0.87 ± 0.2, 0.12 ± 0.05, 0.17 ± 0.05 respectively, much more reduction of tumor volume and packed cell volume were observed in case of diphenylmethyl selenocyanate plus CP treated groups. It has been noted that mean ± (SD) of viable tumor cell counts was 27.6 ± 2.5 in EAC control group (Gr. II). After administration of CP (Gr. III), diphenylmethyl selenocyanate (Gr. IV, Gr. V) and a combination of both CP and diphenylmethyl selenocyanate (Gr. VI, Gr. VII) the mean ± (SD) viable tumor cell counts were 13.5 ± 0.8, 17.1 ± 0.8, 19.1 ± 1.0, 7.0 ± 0.3, 11.1 ± 0.7 respectively, much more reduction of viable tumor cell counts were observed in case of diphenylmethyl selenocyanate plus CP combined treated groups. Control EAC tumor-transplanted mice survived for 23 days. The survival time significantly ($P < 0.05$) increased to 35 (Gr. III), 29 (Gr. IV) and 28 (Gr. V) days with CP and diphenylmethyl selenocyanate alone treated groups. In the groups treated with diphenylmethyl selenocyanate and CP in combination a further significant ($P < 0.05$) increase in survival up to 62 (Gr. VI) and 55 (Gr. VII) days was observed. The increase in the lifespan of tumor bearing mice treated with CP and diphenylmethyl selenocyanate itself was 52.1 (Gr. III), 26.0 (Gr. IV) and 21.7% (Gr. V) respectively. Combination of CP and DMSE increases lifespan much more significantly by 169.5 (Gr. VI) and 139.1% (Gr. VII) respectively.

Apoptosis enhancement effect of diphenylmethyl selenocyanate along with CP on EAC cell was confirmed by tunnel assay (Table 2). Apoptotic index (AI) in the EAC control group was 6.2 ± 1.1 (Gr. II), which was increased significantly after treatment with CP and DMSE and the

Table 2 Effect of diphenylmethyl selenocyanate along with CP on tumor growth, mean survival time, % ILS and apoptosis

Groups	Tumor volume (ml)	Packed cell volume (ml)	Total viable tumor cell count ($\times 10^6$)	Mean survival time (days)	% increased life span (% ILS)	Apoptotic index (%)
II	2.5 ± 0.3	1.5 ± 0.4	27.6 ± 2.5	23 ± 0.8	–	6.2 ± 1.1
III	0.4 ± 0.05^b	0.27 ± 0.09^b	13.5 ± 0.8^b	35 ± 3.4^b	52.1	24.9 ± 1.8^b
IV	1.6 ± 0.2^b	0.75 ± 0.2^b	17.1 ± 0.8^b	29 ± 1.1^b	26.0	13.6 ± 2.1^b
V	1.7 ± 0.08^b	0.87 ± 0.2^b	19.1 ± 1.0^b	28 ± 1.6^b	21.7	12.6 ± 1.6^b
VI	0.2 ± 0.1^c	0.12 ± 0.05^c	7.0 ± 0.3^c	62 ± 5.4^c	169.5	64.0 ± 6.1^c
VII	0.3 ± 0.05^c	0.17 ± 0.05^c	11.1 ± 0.7^c	55 ± 3.4^c	139.1	43.1 ± 4.0^c

Values are expressed as mean ± SD, $n = 6$

[b] $P < 0.05$ statistically compared with Gr. II (EAC control)

[c] $P < 0.05$ statistically compared with Gr. III (CP only treated group)

values were 24.9 ± 1.8 (Gr. III), 13.6 ± 2.1 (Gr. IV) and 12.6 ± 1.6 (Gr. V) but much more pronounced enhancement of apoptotic induction was observed in combined treatment with diphenylmethyl selenocyanate and CP and AI was found to be 64.0 ± 6.1 (Gr. VI) and 43.1 ± 4.0 (Gr. VII) respectively.

Discussion

ROS generated from oxidation–reduction reaction or from activated oxygen species mediated reactions are responsible for a wide range of chemotherapy induced side effects [29]. Selenium has become widely recognized as a nutritional antioxidant and shown to help cancer chemotherapy treatment by enhancing the efficacy of the anticancer drug treatment and reducing the toxicity imparted by the chemotherapeutic drugs [30, 31].

Cancer cells can generate large amounts of hydrogen peroxide which may contribute to their ability to mutate and damage normal tissues, and moreover, facilitate tumor growth and invasion [32]. A number of in vivo and in vitro studies have indicated that tumor growth can cause antioxidant disturbances and induction of lipid peroxidation in tumor hosts [33]. CP administration also results in the build up of free radicals mediated oxidative stress, which leads to inactivation of membrane bound enzymes and induction of lipid peroxidation [34]. The present study also shows that the enhancement in lipid peroxidation level is accompanied by concomitant decrease in the level of reduced glutathione and as well as the activities of the antioxidant enzymes, namely GST, GPx, SOD, CAT in the CP treated animals which is an indication of oxidative stress induced by CP. The significant decrease in lipid peroxidation level coupled with the enhancement of reduced glutathione and antioxidant enzymes by treatment with the selenium compound indicates its role in reducing CP induced oxidative stress.

GSH plays an important role in the detoxification of xenobiotics and in the scavenging of ROS and free radicals, and a decline in its cellular level has been considered to be indicative of oxidative stress mediated cellular damage produced by CP metabolites [35]. The depletion of thiol content observed in CP treated mice liver and lung may also be due to the direct conjugation of 4-hydroxycy-CP (4-OHCP) with cellular thiols to form an alkyl-thio derivative. The alkyl-thio derivative thus formed exists in equilibrium with 4-OHCP and can act as a storage point for alkylating activity [35]. It has been suggested that the reactions of 4-OHCP with various thiol compounds enhance the stability of its metabolites in plasma, as well as facilitate their entry into cells [36]. The protective effect of diphenylmethyl selenocyanate against CP induced toxicity is may be in part due to the reduction of 4-OHCP level resulting in formation of alkyl-thio derivative. Another possible mechanism of protection may be by its ability to deliver phosphoramide mustard to the appropriate targets by increasing the thiol content of the cell. Antioxidant enzyme GST, catalyses the detoxification of endogenous compounds such as lipid peroxide, as well as the metabolites of xenobiotics like acrolein from CP through cojugation of GSH via the sulfhydryl group with these toxic compounds [37]. The increase in GST activity by diphenylmethyl selenocyanate treatment in liver and lung tissue significantly improves the host defense system by catalysing the conjugation of GSH with toxic metabolites from CP. SOD, CAT, and the seleno enzyme GPx are involved in the clearance of superoxide, H_2O_2, as well as lipid peroxides thereby reducing oxidative stress. Therefore enhancement of theses enzymes levels by diphenelmethyl selenocyanate improves the host defense system of the animals during CP treatment.

Myelosuppression and anemia are the most common adverse effects of chemotherapy [38]. Chemotherapeutic drugs kill rapidly dividing cells in the body, including cancer cells and normal cells which include red blood cells, at

the same time suppress bone marrow's ability to produce new ones, resulting decrease in blood hemoglobin level. Treatment with diphenylmethyl selenocyanate brought back the hemoglobin level, RBC, and WBC count more or less to near normal levels, indicating the protective effects of diphenylmethyl selenocyanate on the hemopoietic system. ALT and AST are two marker enzymes for assessment of liver function. If the liver is injured, the liver cells spill the enzymes into blood, raising the enzyme levels in blood and signaling liver damage such as liver necrosis and inflammation. CP treatment of EAC bearing mice significantly elevated the levels of theses transaminases the reason may be due to the formation of toxic metabolites specially acrolein [39] from CP which damaged the liver cells and released these transaminases in to blood. The selenium compound significantly reduced the level of these transaminase activities. This might be due to the scavenging activity of diphenylmethyl selenocyanate against the toxic metabolite that was produced during the activation of the CP by liver microsomal enzymes.

It has been reported that chemotherapy induced ROS generation diminish the efficacy of the cancer treatment by interfering with anticancer drug induced apoptosis, which is important for chemotherapeutic agents to exert their optimal effect on cancer cells [40]. It is thought that death by apoptosis is physiologically advantageous because apoptotic cells are cleared by phagocytosis and subsequent intracellular degradation and thus apoptotic cells are cleared without causing damage to the surrounding tissue. So agent that modulate the ability of antineoplastic drugs to induce apoptosis may enhanced drug efficacy, and prevent tissue damaging inflammatory response. In the present study diphenylmethyl selenocyanate significantly modulate the ability of CP to induce apoptosis as revealed from the significant enhancement of apoptotic index in the groups treated with diphenylmethyl selenocyanate and CP in combination as compared to only CP treated group. This effect was inline with the observation that diphenylmethyl selenocyanate along with CP significantly arrested the tumor growth by decreasing the nutritional fluid volume and viable cell count and the effect was much more than only with CP treatment. The present study also shows a very significant increase in survival of tumor bearing mice treated with diphenylmethyl selenocyanate and CP in combination as compared with treatment using only CP. The results clearly demonstrated that diphenylmethyl selenocyanate does not interfere with the antitumor efficacy of CP on the other hand administration of diphenylmethyl selenocyanate in CP treated animals synergistically enhanced the therapeutic efficacy of CP.

The mechanism of such selective protection of bone marrow cells versus tumor cell growth by diphenylmethyl selenocyanate against CP toxicity is not known. These differences may be due to a number of mechanisms [41] including, (1) differential accumulation of antioxidants by normal and cancer cells, (2) differing requirements for oxidative stress in tumor versus normal cells, with reduction of ROS in cancer cells possibly leading to proliferation inhibition and increased apoptosis, (3) modulation of cellular uptake of drug with some pathways linked to enhanced cell survival and others to cell death depending upon cell type, (4) differential signaling mechanism in cytoprotection of normal cells versus cytotoxicity in tumor cells. The selenium compound was found to be non-toxic at the dose used for the experiment and also at higher doses as seen from the changes of body weight and hair texture [11]. The LD_{50} was found to be above 500 mg/kg body weight [11]. It was also observed that this compound did not have any adverse effect on the hepatic lipid peroxidation and also on the serum ALT and AST level in normal mice (unpublished data).

In summary, the present findings suggest that treatment with diphenylmethyl selenocyanate, a synthetic organic selenium compound protects against CP induced oxidative stress and cellular toxicity in part by upregulating the antioxidant enzymes and GSH levels, modulating serum AST, ALT levels and also normalizing the hematological parameters. It is also evident from this study that diphenylmethyl selenocyanate has the potential to act both as a chemoprotector and tumor growth inhibitor. Further investigations are required to fully explore the exact molecular mode of action of diphenylmethyl selenocyanate on CP induced toxicity and antitumour efficacy.

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