RESEARCH COMMUNICATION

Inhibition of DMBA-croton Oil Two-stage Mouse Skin Carcinogenesis by Diphenylmethyl Selenocyanate Through Modulation of Cutaneous Oxidative Stress and Inhibition of Nitric Oxide Production

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Abstract

Selenium, an essential micronutrient, plays important roles against different diseases, including several types of cancer. In the present study, antioxidative and chemopreventive properties of a synthetic organoselenium compound, diphenylmethyl selenocyanate, were evaluated with a 7,12-dimethylbenz (a) anthracene - croton oil induced two-stage mouse skin carcinogenesis model.

The compound was administered orally to carcinogen-treated mice at two different non-toxic doses, 2mg/kg. b.w. and 3mg/kg. b.w. Significant inhibition in the incidence of papilloma formation (53-80%) as well as in the cumulative numbers of papillomas per papilloma bearing mouse were observed in the treated groups as compared to the carcinogen control group. The compound was also found to upregulate significantly different phase II detoxifying enzymes such as glutathione-S-transferase ($p<0.01$) and superoxide dismutase ($p<0.01$) in skin cytosol when measured after 15 days and also after 12 weeks of the first 7,12-dimethylbenz (a) anthracene treatment. Lipid peroxidation measured with reference to thiobarbituric acid reactive substances in skin microsomes was significantly inhibited ($p<0.05$) in a dose dependent manner by diphenylmethyl selenocyanate. Considerable inhibition of the level of nitric oxide production in peritoneal macrophages was observed after 12 weeks ($p<0.05$).

Thus the compound appears to exert chemopreventive activity in terms of papilloma formation, which may be through modulation of cutaneous lipid peroxidation, the phase II detoxifying enzyme system and nitric oxide production.

Key Words: 7,12-dimethylbenz (a) anthracene- croton oil - diphenylmethyl selenocyanate - phase II enzymes - lipid peroxidation - nitric oxide

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Introduction

Though recent advances in cancer detection and therapy have improved the short-term prognosis and quality of life of cancer patients, the improvement is only slight in terms of the mortality rates for most cancers. Such limitations in cancer treatment have led to interest in developing approaches that may prevent the onset of cancer. In this effort, a large number of chemopreventive agents have been identified which afford protection, as demonstrated by animal model studies and epidemiological surveys (Boone et al., 1990; Wargovich, 1997).

Epidemiological studies and human intervention trials indicate that selenium may have chemopreventive activity in human beings. In laboratory animal assays, chemopreventive effects of inorganic and organic selenium compounds have been observed in mammary gland, colon, lung, pancreas and skin (Sugie et al., 2000) at all stages, initiation, promotion and progression. Different organoselenium compounds were found to have the ability to reduce hydrogen peroxide, lipid and phospholipid hydroperoxide levels, thereby dampening the propagation of free radicals; reactive oxygen and nitric oxide species mediating cellular damage (Muller et al 1984; Matsumoto

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et al., 2001). Selenium supplementation in diet can reduce malonaldehyde production and inducible nitric oxide synthase (iNOS) activity (Ksng et al., 1998; Hattori et al 1994). Modulatory roles of selenium in the inactivation of NF-kB and COX-2 activity have also been reported. Several inorganic or organic forms of selenium have received wide attention as possible cancer chemopreventive agents (Fishbein, 1986a; 1986b; McGarrity et al., 1993; Shamberger, 1970; 1986).

DMBA is a ubiquitous environmental pollutant with high carcinogenic potential that is considered one of the etiological factors for human cancers through its presence in cigarette smoke and environmental mixtures. It is metabolically activated in cells by cytochrome p450 enzymes to reactive intermediates that damage DNA.

Tumour promoters stimulate hydrogen peroxide and super oxide production. Oxidative mechanism plays a determining role in activating and amplifying the process of tumour promoters.7,12-dimethylbenz (a) anthracene induced and croton oil promoted mouse skin carcinogenesis is a useful murine model for study of genetic and biological changes involved in tumor promotion, and also for screening of new cancer protective agents, as the model displays a preneoplastic condition during carcinogenesis in the form of papillomas, which are visible and can be confirmed histopathologically.

In a previous report we provided evidence of chemopreventive efficacy of diphenylmethyl selenocyanate, a synthetic organoselenium compound, during two-stage skin carcinogenesis in mice (Das et al., 2004). The present study was undertaken to explore the in vivo action of the compound, focusing on nitric oxide production by peritoneal macrophages, suppression of membrane lipid peroxidation in mouse skin and upregulation of cutaneous phase II antioxidant enzymes in a DMBA-croton oil two-stage mouse skin carcinogenesis model, with the aim of throwing light on possible mechanisms of action.

Materials and Methods

Chemicals

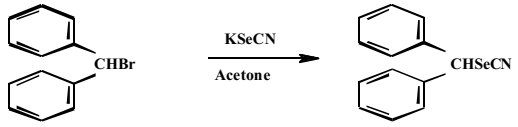
7,12 dimethylbenz (a) anthracene (DMBA), 1-chloro-2, 4-dinitrobenzene (CDNB), ethylene diamine tetra acetic acid (EDTA), Reduced Glutathione (GSH), Pyrogallol, sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), bovine serum albumin (BSA), thioglycolate broth were obtained from Sigma (St.Louis, M.O., USA). Hydrogen peroxide 30% (H_2O_2), sodium nitrite were obtained from "Merck Limited" (India), RPMI 1640, fetal calf serum (FCS) were obtained from Hyclone Laboratories, Logan, UT.

Animals

Adult (5-6 weeks) Swiss albino female mice (22_2gm.), 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.

Synthesis of the Compound

Diphenylmethyl selenocyanate was prepared following a published procedure (Pederson, 1963). Briefly, diphenylmethyl bromide was treated with potassium selenocyanate (KSeCN) in acetone at 60-70 ^{0}C for 5 hours. 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. 68-69^0 C.



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Drug Preparation

Synthetic organoselenium compound diphenylmethyl selenocyanate was used as a suspension in 5.5% propylene glycol in water, prepared each day of experiment, just before treatment.

Experimental Design

Mice were divided into eight groups (Gr. I- VIII). Gr. I, II and III comprised of 10 mice each and Gr. IV to VIII had 20 mice each, 10 mice for 15 days experimental protocol and the other 10 for 12 weeks experimental protocol.

The back of the animals of Gr. IV, V, VI, VII and VIII were shaved 2 days prior to the start of the experiment.

Gr. I: Normal mice were given only propylene glycol (5.5% in water) 300 µl/mouse/day by oral gavages for 12 weeks.

Gr. II and III: Normal animals were treated with the drug at a dose of 2mg/kg. b.w. and 5 mg/ kg. b.w. respectively for 12 weeks.

Gr. IV animals received topical application of acetone (100µl/mouse) on the shaved skin and propylene glycol (300µl/mouse) by oral gavage for 15 days and 12 weeks and served as vehicle controls.

Gr. V animals received two topical application of 7,12 dimethylbenz (a) anthracene at an interval of 72 hrs, at a dose of 0.05g/kg b.w. in acetone (100 µl/mouse), followed by croton oil (1% w/v) in acetone (100 µl/mouse), twice in a week for 9 weeks starting from day 8 of 1st 7,12 dimethylbenz (a) anthracene application and served as carcinogen control.

The animals of group VI and VII received the same treatment as for gr V and also received the selenium compound at a dose of 2mg/kg b.w. and 3 mg/kg b.w. respectively from the day of 7,12 dimethylbenz (a) anthracene treatment.

Gr. VIII served as a pretreatment group. This group was also given the same treatment as for gr V except that it received an oral administration of the selenium compounds

at a dose of 2mg/kg b.w. for 15 days before the 1st 7,12 dimethylbenz (a) anthracene application which was continued throughout the experimental period.

During the experiment, mice of grs I to VIII were weighed weekly until the time of autopsy (data not shown).

Mice of group IV, V, VI, VII, VIII were sacrificed following the guidelines as mentioned by the ethical committee of our Institute (Regn. no- IAEC-1.2/SD1/2001-2003) after 15 days and 12 weeks of 1st 7,12 dimethylbenz (a) anthracene application and the parameters described below were studied. The experiments were performed in triplicate.

Dose Selection

The compound was administered orally to normal mice (gr. II and III) in different doses and the changes in body weight were calculated at weekly interval upto 12 weeks. No significant changes were note in the body weight of Gr. I and the drug treated (gr. II and gr. III) mice. A loss of body weight was observed among the mice treated with doses above 5mg/kg. b.w. as weight loss may be considered as a criteria of toxicity. Two doses (2mg/kg. b.w. and 3mg/kg.b.w.), below 5mg/kg. b.w. were then considered for the present experiment and the experimental groups were distributed accordingly (data not shown).

Detection of Papillomas

The experimental animals were carefully examined once a week for counting and recording the incidence of papilloma and the number of papilloma per papilloma bearing mouse from 6th weeks onwards. Skin papillomas with a diameter greater than 1mm that persists for at least two consecutive observations were used for counting. The papillomas, which regressed after one observation, were not considered for counting. Two different experts who were not supplied with information regarding the experimental groups performed the measurement of papillomas.

Tissue Preparation

Preparation of Skin Microsomes for Quantitative Estimation of Lipid Peroxidation

Skin tissues of the animals were collected, washed in 0.9% saline, soaked in filter paper, weighed and 400mg skin tissue were homogenized in 1.15% KCl. The homogenates were then centrifuged at 12000g at 4^0C for 10 minutes, the supernatant were collected, and again centrifuged at 10,000g at 4^0C for 10 minutes. This supernatant was then ultra centrifuged at 54,000g at 4^0C for 1 hr. The resultant supernatant was discarded and the microsomal pellet was dissolved in 0.4 ml. 1.15% KCl to give the samples for assay of lipid peroxidation.

Preparation of Skin Cytosol for Glutathione-S-Transferase Assays

Skin tissues of experimental animals were collected, washed in 0.9% saline, soaked in filter paper and weighed. Tissue fragments (200mg) were homogenized in cold condition in 1ml homogenizing buffer (250 mM sucrose, 20mM Tris-HCl, 1mM dithiothreitol, pH 7.4), using glass-Teflon homogenizers. The homogenates were centrifuged at 75,000 g at 4^0C for 2hr. Supernatant (cytosolic fraction) was separated into aliquots and stored until used for the assay (Mulder et al., 1979).

Preparation of Skin Cytosol for Superoxide Dismutase Assays

Skin tissues of the animals were collected, washed in 0.9% saline, soaked in filter paper, weighed. 100mg skin tissue from each animal was homogenized in 0.5ml of Tris-HCl buffer and the homogenate was centrifuged at 10,000g for 30 mins at 4^0C. The supernatant was taken and 0.25 ml of ethanol and 0.15 ml of chloroform was added per ml of the supernatant, vortexed for 5-7mins and centrifuged at 13,000g for 15 mins and the supernatant used as the enzyme source.

Preparation of Macrophage Cultures for Assays of Nitrite Production in Peritoneal Macrophages

Peritoneal macrophages were elicited in all animals by intraperitonial injection of 2ml of 4% thioglycolate broth. Four days after injection, macrophages were harvested by peritoneal lavage using sterile phosphate buffered saline (PBS). Cells were washed, centrifuged and $2x\ 10^5$ cells/well were cultured in 96 well flat bottom titre plates in RPMI-1640 supplemented with 10% heat inactivated FCS for 2hr at 37^0C in 5% CO_2. After incubation the nonadherent cells were removed by aspiration and freshly prepared complete medium was added and incubated for 24hr at 37^0C in 5% CO_2. Then the cell -conditioned medium was collected after 24 hr for assay of nitrite (NO_2^-) production in the cell free supernatant (Ding et al., 1988).

Biochemical Estimation

Quantitative Estimation of Cutaneous Lipid Peroxidation

Lipid peroxidation was estimated in skin microsomal fraction. The level of lipid peroxides formed was measured using thiobarbituric acid and expressed as nano mole of thiobarbituric acid reactive substances (TBARS) formed per mg of protein using extinction co-efficient of $1.56x10^5\ M^{-1}cm^{-1}$ (Okhawa et al., 1974).

Estimation of Cutaneous Glutathione - S - Transferase (GST) Activity

Glutathione -S-Transferase (GST) activity was measured in the skin cytosol. The enzyme activity was determined from the increase in absorbance at 340nm with 1-chloro-2-4-dinitrobenzene (CDNB) as the substrate and specific activity of the enzyme expressed as formation of 1-chloro-2-4-dinitrobenzene (CDNB)-GSH conjugate per minute per mg of protein. (Habig et al., 1974).

Estimation of Cutaneous Superoxide Dismutase (SOD) Activity

SOD activity was assayed by the method of Marklund

et al. (Marklund et al., 1974) and the partial extraction and purification of SOD was done as described by Mc Cord et al. (McCord et al., 1969). Superoxide dismutase (SOD) activity was determined by quantification of Pyrogallol auto oxidation inhibition and expressed as unit/mg of protein. One unit of enzyme activity is defined as 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. .

Assay of Nitrite Production in Peritoneal Macrophages

Nitric oxide (NO) produced by macrophages quickly reacts with oxygen to produce nitrate (NO_3^-) and nitrite (NO_2^-) ions. Accumulation of nitrite in the medium was measured spectrophotometrically based on Griess reaction (Green et al., 1981). In brief, 50 µl of Griess reagent mixture of 0.1% N- (1-napthyl) ethylenediamine dyhydrochloride, 1% sulfanilamide and 2.5% orthophosphoric acid was reacted with 50µl of sample (cell free supernatant) at room temperature for 10 min and the NO_2^- concentration was determined by absorbance at 550nm in comparison with the sodium nitrite ($NaNO_3$) standards. Data were represented as µM of nitrite produced in 50µl cell free supernatant.

Estimation of Protein

Protein was estimated spectrophotometrically (Lowry et al., 1951) with bovine serum albumin as the standard.

Statistical Analysis

The data was analyzed by ANOVA followed by Tukey's test [SYSTAT 9.0; SPSS Inc., Chicago, USA] to identify the differences between the means of different groups. A p value of <0.05 was considered significant.

Results

Effect of Diphenylmethyl Selenocyanate on DMBA Induced Croton Oil Promoted Incidence and Average Number of Skin Papillomas

The incidence of papilloma in 7,12 dimethylbenz (a) anthracene - croton oil treated and 7,12 dimethylbenz (a) anthracene - croton oil - selenium compound treated groups at different weeks is shown in Fig. 1. The incidence in 7,12 dimethylbenz (a) anthracene -croton oil treated mice (gr. V) reached 100% after 12 weeks of experiment. Mice of gr. VI that received selenium compound throughout the initiation stage of the experiment at a dose of 2mg/kg b.w. showed a tumor incidence of 46.32%, whereas animals of gr. VII receiving the drug at a dose of 3mg/kg b.w. showed 20% tumor incidence. Animals of gr. VIII given the selenium compound at pre initiation as well as the post initiation phase showed an incidence of papilloma of 40%. The values are significantly lower ($p<0.01$) than the carcinogen control group (gr. V) (Fig 1).

The number of papillomas per papilloma bearing mouse in the drug treated group were also significantly less ($p<0.01$) compared to the carcinogen control group. The mean number

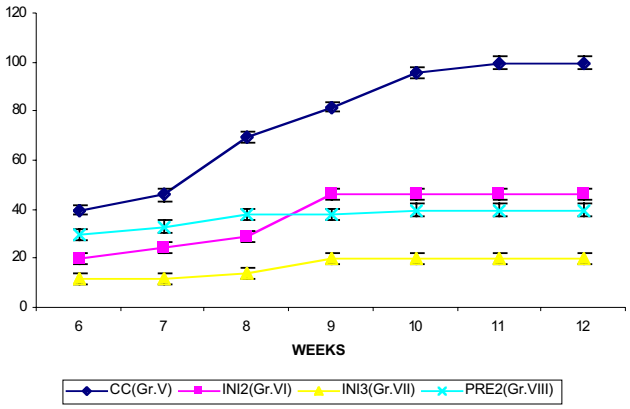

Figure 1. Percentage Incidences of Papillomas in Different Groups After 12 Weeks

of papillomas per papilloma bearing mouse are 1.61±0.09 for gr. VI, 1.19±0.18 in gr. VII and 1.40±0.09 in gr. VIII whereas that in gr. V was 3.93±0.49 (Fig: 2).

Effect of Diphenylmethyl Selenocyanate on Survival of Mice

Survival of mice that received only carcinogen (gr. V) decreased significantly ($p<0.01$) compared with vehicle treated group (gr. IV). Significant improvement was noted ($p<0.05$) when the animals were given selenium compound (gr. VI, VII, VIII) as compared to the animals treated with DMBA-croton oil alone (gr. V) (Fig 3).

Biochemical Studies in Mouse Skin

Lipid Peroxidation Level in Skin Microsomes

The level of lipid peroxidation in skin of the animals treated with 7,12 dimethylbenz (a) anthracene - croton oil (gr. V) increased significantly ($p<0.01$) by 99.06% after 12 weeks of treatment compared to vehicle treated animal (gr. IV). The level of lipid peroxidation was decreased significantly ($p<0.01$) by 49.28% (gr. VI), 58.63% (gr. VII) and 80.57% (gr. VIII) after 12 weeks of treatment by the selenium compound as compared to the level in 7,12 dimethylbenz (a) anthracene - croton oil treated animals (gr. V) (Table 1). No significant alteration in lipid peroxidation was noted after 15 days of treatment in the skin.

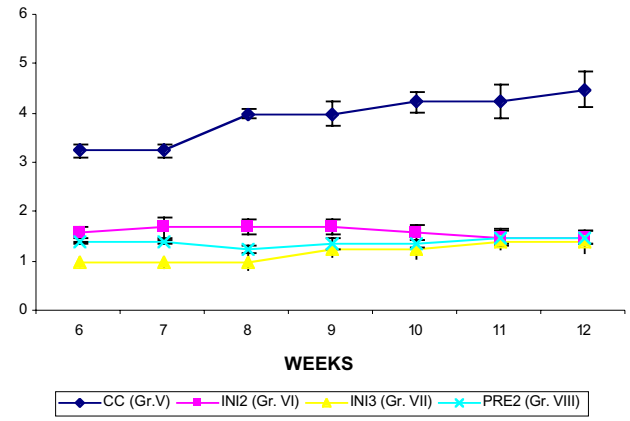

Figure 2. Cumulative Numbers of Papillomas per Papilloma Bearing Mouse After 12 Weeks

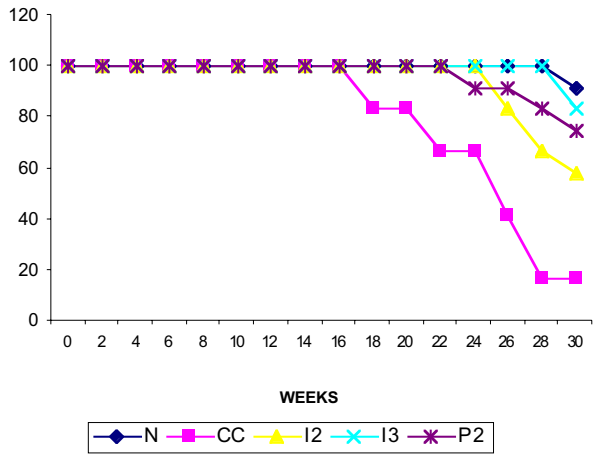

Figure 3. Survival of Mice After 30th Weeks in Different Groups

Glutathione -S-Transferase Activity

As compared with the vehicle treated animals (gr. IV) the activity of glutathione-S-transferase in the skin of animals treated with 7,12 dimethylbenz (a) anthracene - croton oil (gr. V) showed a decrease of 58.56% and 85.62% ($p<0.01$) after 15 days and 12 weeks of 7,12 dimethylbenz (a) anthracene treatment respectively. On treatment with diphenylmethyl selenocyanate, the Glutathione -S-transferase activity rose sharply by 34.78% (gr. VI), 66.66% (gr. VII) and 55.08% (gr. VIII) when measured after 15 days of 1st 7,12 dimethylbenz (a) anthracene treatment compared to the 7,12 dimethylbenz (a) anthracene - croton oil control (gr. V). This elevated level continued till the end of the experiment (12 weeks) and a 48.88% (gr. VI), 62.29% (gr. VII) and 59.64% (gr. VIII) increase ($p<0.01$) in the Glutathione -S- transferase level was observed in the selenium compound treated groups in comparison to the carcinogen control group (gr. V) (Table 2).

Superoxide Dismutase Activity

A significant ($p<0.01$) decrease of 65.19% and 52.05% in the level of this enzyme activity was observed in 7,12 dimethylbenz (a) anthracene - croton oil treated animals (gr. V) in comparison to the vehicle treated mice (gr. IV) after

Table 1. Modulation of Skin Microsomal Lipid Peroxidaion and Inhibition of Overproduction of Nitric Oxide from Peritoneal Macrophages by Diphenylmethyl Selenocyanate in DMBA-Croton Oil Two-stage Mouse Skin Carcinogenesis After 12 Weeks

GROUPS	Lipid peroxidation (nmol of thiobarbituric acid reactive substances/ mg of protein)	Nitric oxide (NO) (_M/50_l)
IV	0.026 ± 0.002	3.10 ± 0.13
V	2.78 ± 0.35 *	6.94 ± 0.43 *
VI	1.41 ± 0.21 *	4.09 ± 0.55 *
VII	1.15 ± 0.13 *	1.55 ± 0.26 *
VIII	0.54 ± 0.07 *	3.34 ± 0.17 *

Data represent the Mean _SE. * $p<0.01$; Comparisons are made in the text between
1. Gr. IV (vehicle control) and Gr. V (DMBA+Croton oil treated carcinogen control group)
2. Gr. V (Carcinogen control) and Gr. VI (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 12 weeks)
3. Gr. V (Carcinogen control) and Gr. VII (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 12 weeks)
4. Gr. V (Carcinogen control) and Gr.VIII (15 days pretreatment at a dose of 2mg/kg. b.w + DMBA-croton oil + post treatment at a dose of 2mg/kg. b.w for 12 weeks)

Table 2. Modulation of Cutaneous Phase II Detoxifying Enzyme Activity by Diphenylmethyl Selenocyanate in DMBA-Croton Oil Two-stage Mouse Skin Carcinogenesis After 15 Days and 12 Weeks

GROUPS	GST (CDNB-GSH conjugate/min/mg of protein)		SOD (unit/mg of protein)	
	15 DAYS	12 WEEKS	15 DAYS	12 WEEKS
IV	0.181 ± 0.007	0.160 ± 0.04	38.50 ± 1.2	40.36 ± 0.15
V	0.075 ± 0.01*	0.023 ± .001*	13.40 ± 0.77*	19.35 ± 1.46*
VI	0.115 ± 0.02**	0.045 ± 0.002*	71.14 ± 3.4*	63.33 ± 2.88*
VII	0.225 ± 0.02*	0.061 ± 0.008*	105.35 ± 1.25*	104.14 ± 3.74*
VIII	0.167 ± 0.01*	0.057 ± 0.004*	78.20 ± 2.27*	93.06 ± 1.71*

Data represent the Mean ±SE. * $p<0.01$; ** $p<0.05$. Comparisons are made in the text between
1. Gr. IV (vehicle control) and Gr. V (DMBA+Croton oil treated carcinogen control group)
2. Gr. V (Carcinogen control) and Gr. VI (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 15 days and 12 weeks)
3. Gr. V (Carcinogen control) and Gr. VII (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 15 days and 12 weeks)
4. Gr. V (Carcinogen control) and Gr.VIII (15 days pretreatment at a dose of 2mg/kg. b.w + DMBA-croton oil + post treatment at a dose of 2mg/kg. b.w for 15 days and 12 weeks)

15 days and 12 weeks of the experiment. This level were significantly ($p<0.01$) elevated by 81.20% (gr. VI), 87.28% (gr. VII) and 82.86% (gr. VIII) in all the selenium compound treated group after 15 days of treatment. Significant ($p<0.01$) increase of 69.44% (gr. VI), 81.41% (gr. VII) and 79.20% (gr. VIII) is also noted in the treated group as compared to carcinogen control group (gr. V) when measured after 12 weeks of carcinogen treatment (Table 2).

Effect of Diphenylmethyl Selenocyanate on Nitrite Production in Peritoneal Macrophages

The nitrite level of the animals treated with 7,12 dimethylbenz (a) anthracene - croton oil (gr. V) increased significantly ($p<0.01$) by 55.33% after 12 weeks of treatment compared to vehicle treated animal (gr. IV). The level of nitrite production was decreased significantly ($p<0.01$) by 41.06% (gr. VI), 77.66% (gr. VII) and 51.87% (gr. VIII) after 12 weeks of treatment with the selenium compound as compared to the level of 7,12 dimethylbenz (a) anthracene - croton oil treated animals (gr. V) (Table 1).

Discussion

Murine skin carcinogenesis is a stepwise process, consisting of initiation, promotion and progression (Agarwal and Mukhtar, 1998). DMBA is metabolized by Cyp1A1 and Cyp1B1, the two enzymes of the Cyp 450 family (Kawajiri, 1999; Angus et al., 1999 and Buters et al., 1999) to the ultimate carcinogen 1,2-epoxide-3, 4-diol DMBA, which form adducts with DNA (Dipple et al., 1984; Cheng et al., 1988a and Cheng et al., 1988b). These adduct lead to mutations, which are a prerequisite for the development of tumors (Dipple et al., 1999; Shelton et al., 2000). The tumour promotion stage; clonal expansion and selective hyperplasia of initiated keratinocytes can be triggered by repeated application of croton oil, which is rich in phorbol esters, ultimately producing a squamous papilloma (Allen et al., 2003 and Slaga et al., 1996). The tumour progression is characterized by high level of genetic instability that leads to chromosomal alterations (Warren et al., 1993).

The present study demonstrates that, the incidence and number of skin papillomas initiated with 7,12 dimethylbenz (a) anthracene and promoted by croton oil can be significantly decreased by treatment with diphenylmethyl selenocyanate in Swiss albino female mice. Maximum inhibition was noted at the dose of 3mg/kg. b.w. (gr. VII). This may be due to several factors such as inhibition of 7,12 dimethylbenz (a) anthracene metabolism to its active form or delay in the promotion phase of carcinogenesis by down regulating production of reactive oxygen species, iNOS regulated overproduction of NO induced papillomagenesis and lipid peroxidation or modulation of ornithine decarboxylase, protein kinase C activity or by decreasing prostaglandin synthesis (Kausar et al., 2003) by the selenium compound. The survival of mice in the selenium compound treated group also increased after 30th weeks than the carcinogen control group. This effect could be associated with the low tumour burden as a result of the inhibitory effect of selenium compound on papillomagenesis in the treated group. Maximum survival (83.33%) of mice was noted in the group treated with the compound at a dose of 3mg/kg.b.w. (gr. VII) (Fig: 2).

Repeated topical application of the tumour promoter Croton oil on mouse skin involves both oxidative burst as well as inflammation by stimulating the generation of nitric oxide (NO), reactive nitric oxide species (Ahmead et al., 1997) and reactive oxygen species from Langerhans cells, polymorphonuclear leukocytes, macrophage and non phagocytic cells (Sing et al., 1985; Tamir and Tannenbaum, 1996). ROS plays an important role in the process of mutagenesis and carcinogenesis particularly in tumour promotion. They can induce lipid peroxidation, DNA strand break by modulating different biochemical pathways and gene expression (Halliwell, 1999). It has been suggested that iNOS mediated overproduction of NO is often observed during inflammation (MacMicking et al., 1997) and tumour development (Rao et al., 1999) by enhancing DNA damage, lipid peroxidation and decreasing cellular antioxidant store and influence the process of multistage carcinogenesis (Tamir and Tannenbaum, 1996). The enhanced level of thiobarbituric acid reactive substances in skin of animals treated with 7,12 dimethylbenz (a) anthracene - Croton oil after 12 weeks suggests oxidative stress in 7,12 dimethylbenz (a) anthracene - Croton oil induced mouse skin carcinogenesis. The significant decrease in lipid peroxidation levels by treatment with the selenium compound indicates its role in reducing oxidative stress. After 12 weeks of experiment maximum effect was observed for group VIII that received the selenium compound at a dose of 2mg/kg.b.w. in a pre+post treatment schedule.

Several recent reports demonstrate that NO couples to superoxide anion (O_2^-) and produced peroxinitrite ($ONOO^-$) and stimulate prostaglandin biosynthesis *in vivo*, in perfused organ and in macrophages (Landino et al., 1996; Salvemini et al., 1993). Peroxynitrite ($ONOO^-$) can damage DNA (Weiming et al., 2002), inhibit variety of enzymes and initiate membrane lipid peroxidation (Radi et al., 1991) and depletion of antioxidant enzyme and glutathione stores (van der Vliet et al., 1994; Tamir and Tannenbaum, 1996). On the other hand NO undergoes autooxidation and leads to the formation of a powerful electrophillic nitrosating agent dinitro trioxide (N_2O_3). The consequence of N_2O_3 formation is important because it leads to formation of N-nitroso compounds and deamination and crosslinking of DNA. N_2O_3 can also inactivate key enzymes involved in protection against oxidative stress and DNA repair process in vivo (Tamir and Tannenbaum, 1996). In the carcinogen control group (gr. V) enhanced production of nitric oxide by peritoneal macrophages was observed after 12 weeks compared to normal control animals, which suggests that nitric oxide plays a part in the DMBA-croton oil induced two stage skin carcinogenesis process. Significant decrease of nitric oxide level from peritoneal macrophages was observed in the selenium compound treatment groups

indicate its role in reducing the generation of nitric oxide may be by inhibiting the expression of iNOS mediated excessive production of nitric oxide and its conversion to strong electrophillic intermediates $ONOO^-$ and N_2O_3 during the development of skin papilloma. The maximum effect was observed at the dose of 3mg/kg.b.w. (gr. VII), which is in accordance with the observation made in the incidence and number of papilloma formation.

Glutathione - S - transferase (GST) plays an important role in detoxifying/transport of active metabolites of carcinogen compounds, by catalyzing the conjugation of GSH with the active metabolites resulting their elimination from the system. It was noted from the experiment that DMBA-croton oil treatment lowered the level of cutaneous GST. Treatment with selenium compound significantly elevated the level of Glutathione -S- transferase activity in skin tissue suggesting the role of diphenylmethyl selenocyanate in improving the host defense system for providing cellular protection through GST mediated neutralization of carcinogenic metabolites.

Super oxide dismutase (SOD) is generally regarded as the first line of antioxidants that protects cells against free radical stress. Literature report reveals that activities of antioxidant enzyme SOD were lowered in squamous cell carcinoma. The decreased level of SOD in papilloma and squamous cell carcinoma leads to pro-oxidant state of cell facilitating tumorigenesis (Oberly et al., 1993). The low level of cutaneous SOD in DMBA - croton oil treated group further confirm the state of oxidative stress. Impaired SOD activity together with decrease level of cellular GST content might make the tissue vulnerable to O_2^- toxicity and lipid peroxidation. Also, it might hamper detoxification capabilities and make the system more prone to toxicity made by reactive nitric oxide species and H_2O_2. Treatment with selenium compound elevates the level of superoxide dismutase activity significantly in the skin tissue of treatment group, which again proves its ability to reduce the propagation of free radicals induced damage.

The present investigation regarding the chemopreventive efficacy of synthetic organoselenium compound diphenylmethyl selenocyanate in a dose dependent manner against DMBA-croton oil two stage skin carcinogenesis process suggest its potential role as an antioxidative as well as anticancer agent.Whether it works through other pathways such as through inhibition of 7,12 dimethylbenz (a) anthracene metabolism by Cyp450 enzymes to its active form or down regulation of the protein kinase - C activity, or inhibition of prostaglandin synthesis, or inactivation of NF-*B thereby reducing iNOS expression or by reducing the level of ornithine decarboxylase activity and polyamine biosynthesis is to be seen.

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References

Agarwal R, Mukhtar H (1998). Cutaneous chemical carcinogenesis. In 'Pharmacology of the Skin' Ed Mukhtar H. CRC Press, Boca Raton, FL. pp. 371-387.

Ahmad N, Srivastava RC, Agarwal R, Mukhtar H (1997). Nitric oxide synthase and skin tumor promotion. *Biochem Biophys Res Commun*, **232**, 328-31.

Allen SM, Florell SR, Hanks AN, et al (2003). Survivin expression in mouse skin prevents papilloma regression and promotes chemical-induced tumor progression. *Cancer Res*, **63**, 567-72.

Angus WG, Larsen MC, Jefeoate CR (1999). Expression of CYP1A1 and CYP1B1 depends on cell-specific factors in human breast cancer cell lines: role of estrogen receptor status. *Carcinogenesis*, **20**, 947-55.

Boone CW, Kelloff GJ, Malone WE (1990). Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials. *Cancer Res*, **50**, 2-9.

Buters JTM, Sakai S, Richter T, et al (1999). Cytochrome p450 CYP1B1 determines susceptibility to 7, 12- dimethylbenz (a) anthracene – induced lymphomas. *Proc Natl Acad Sci USA*, **96**, 1977-82.

Cheng SC, Prakash AS, Pigott MA, et al (1988b). A metabolite of the carcinogen 7, 12 - dimethylbenz (a) anthracene that reacts predominantly with adenine residues in DNA. *Carcinogenesis*, **9**, 1721-3.

Cheng SC, Prakash AS, Pigott MA, et al (1988a). Characterization of 7, 12- dimethylbenz (a) anthracene-adenine nucleoside adducts. *Chem Res Toxicol*, **1**, 216-21.

Das RK, Ghosh S, Sengupta A, Das S, Bhattacharya S (2004). Inhibition of DMBA-croton oil induced two-stage mouse skin carcinogenesis by diphenylmethyl selenocyanate. *European J Cancer Prev*, (in press).

Ding AH, Nathan CF, Stuehr DJ (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J Immunol*, **141**, 2407-12.

Dipple A, Khan QA, Page JE, Ponten J, Szeliga J (1999). DNA reactions, mutagenic action and stealth properties of polycyclic aromatic hydrocarbon carcinogens (review). *Int J Oncol*, **14**, 103- 11.

Dipple A, Moschel RC, Bigger CAH (1984). Polynuclear aromatic hydrocarbons, In 'Chemical Carcinogens' Ed Searle CE. American Chemical Society, Washington DC, pp. 245-314.

Fishbein L (1986a). Perspectives in metal carcinogenesis. I. Selenium. *Arch Geschwuistforsch*, **56**, 53-78.

Fishbein L (1986b). Perspective on selenium anticarcinogenecity. *Toxicol Environ Chem*, **12**, 1-30.

Green LC, de Luzuriaga KR, Wagner DA, et al (1981). Nitrate biosynthesis in man. *Proc Natl Acad Sci USA*, **78**, 7764-8.

Habig WH, Pabst MJ, Jacoby WB (1974). Glutathione-S-Transferases, the first enzymatic step in marcapturic acid formation. *J Biol Chem*, **249**,7130-9.

Halliwell B (1999). Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition. *Mutat Res*, **443**, 37-52.

Hattori R, Inoue R, Sase K, et al (1994). Preferential inhibition of inducible nitric oxide synthase by ebselen. *Eur J Pharmacol*, **267**, R1-R2.

Kawajiri K (1999). CYP1A1, IARC Scientific Publications, Chapter 15, IARC, Lyon, pp. 159-172.

Kang BPS, Bansal MP, Mehta U (1998). Selenium supplementation and diet induced hypercholesterolemia in rat: changes in lipid levels, malonyldialdehyde production and nitric oxide synthase activity. *Gen Physiol Biophys*, **17**, 71-8.

Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ (1996). Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci USA*, **93**, 15069-74.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement with Folinphenol reagent. *J Biol Chem*, **193**, 265-76.

MacMicking J, Xie QW, Nathan C (1997). Nitric oxide and macrophage function. *Ann Rev Immunol*, **15**, 323-50.

Marklund S, Marklund G (1974). Involvement of the superoxide anion radical in the oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Europ J Biochem*, **47**, 469-74.

Matsumoto K, Inagaki T, Hirunuma R, Enomoto S, Endo S (2001). Contents and uptake rates of Mn, Fe, Co, Zn, and Se in Se-deficient rat liver cell fractions. *Analytical Sciences*, **17**, 587-91.

Mc Cord JM, Fridovich I (1969). Superoxide dismutase: An enzymatic function for erythrocuprein (hemoprotein). *J Biol Chem*, **244**, 6049-55.

McGarrity TJ, Peiffer LP (1993). Selenium and difluromethylornithine additively inhibit DMH-induced distal colon tumour formaion in rats fed a fiber-free diet. *Carcinogenesis*, **14**, 2335-40.

Mulder TPJ, Manni JJ, Roelofs HMJ, Peters WHM, Wiersma A (1995). Glutathione-S-transferases and glutathione in human head and neck cancer. *Carcinogenesis*, **16**, 619-24.

M_ller A, Cadenas E, Graf P, Sies H (1984). A novel biologically active seleno-organic compound-I. *Biochem Pharmacol*, **33**, 3235-9.

Oberley TD, Oberley LW (1993). Oxygen radicals and cancer. In Free Radicals in Aging (eds Yu B P); CRC Press, Boca Raton, FL, pp. 247-267.

Okhawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Annal Biochem*, **95**, 351-8.

Pederson CTH (1963). Preparation of some 4-substituted selenosemicarbazides. *Acta Chemica Scandinavica*, **17**, 1459-61.

Radi R, Bcckman JS, Bush KM, Freeman BA (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys*, **288**, 481-7.

Rao CV, Kawamori T, Hamid R, Reddy BS (1999). Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. *Carcinognesis*, **20**, 641-4.

Salvemini D, Misko TP, Masferrer JL, et al (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA*, **90**, 7240-44.

Shamberger RJ (1970). Relationship of selenium to cancer. I Inhibitory effect of selenium on carcinogenesis. *Journal of National Cancer Institute*, **44**, 931-6.

Shamberger RJ (1986). Abstracts of international workshop trace element analytical chemistry in medicine and biology. Abstract No. 19. April 21-23 Gsf-Bericht, Neuherberg, FRG.

Shelton SD, Cherry V, Manjanatha MG (2000). Mutant frequency and molecular analysis of in vivo lacI mutations in the bone marrow of Big Blue rats treated with 7, 12- dimethylbenz (a) anthracene. *Environ Mol Mutagen*, **36**, 235-42.

Singh N, Poirier G, Cerruti P (1985). Tumor promoter PMA induces poly ADP ribosylation in fibroblasts. *EMBO J*, **4**, 1491-4.

Slaga TJ, Budunova IV, Gimenez-Conti IB, Aldaz CM (1996). The mouse skin carcinogenesis model. *J Invest Dermato Symposium Proceedings*, **1**, 151- 6.

Sugie S, Tanaka T, El-Bayoumy K (2000). Chemoprevention of carcinogenesis by organoselenium compounds. *J Health Sc*, **46**, 422-5.

Tamir S, Tannenbaum SR (1996). The role of nitric oxide ($NO^{\cdot}$) in the carcinogenic process. *Biochim Biophys Acta*, **1288**, F31-F36.

van der Vliet A, Smith D, O'Neill CA, et al (1994). Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. *Biochem J*, **303**, 295-301.

Wargovich MJ (1997). Experimental evidence for cancer preventive elements in foods. *Cancer Lett*, **114**, 11-7.

Warren BS, Naylor MF, Winberg LD (1993). Induction and inhibition of tumor progression. *Proc Soc Exp Biol Med*, **202**, 9-15.

Weiming XU, Liu LZ, Loizidou M, Ahmed M, Charles IG (2002). The role of nitric oxide in cancer. *Cell Res*, **12**, 311-20.