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Preliminary Carcinogenic and Cocarcinogenic Studies on Captan Following Topical Exposure in Mice

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Captan (N-trichloromethyl thio-1,cyclohexene-1,2-dicarboximide) is a broad spectrum, nonpersistant fungicide widely used for the control of various fungal diseases of seeds, grains, plants and fruits. It is also used as an industrial fungicide in paints, plastics, leather, soaps and shampoos (NCI,1977). The structure of captan (Fig.1) is closely related to that of thalidomide (Dalvi and Ashley,1979), a potent teratogen in humans, but there are conflicting reports about its teratogenic activity in experimental aninmals. Robens (1970) has shown that captan tratment at high dose levels on day eight of gestation resulted in fused, irregular vertebrae, amelia of right raer leg in golden hamsters. Acute toxicity of captan is reported to be relatively low when given orally, however, it is 200 times as toxic if administerd intraperitoneally (Peeples and Dalvi, 1978) . At the molecular level, it has been found to uncouple oxidative phosphorylation, thus inhibiting cellular respiration (Nelson, 1971) .Absorbed captan, given either orally or i.p., has been found to be very toxic to liver as measured by the inhibition of liver microsomal enzymes and the incidence of ascites (Nelson, 1971,Truhant et al 1974) . Glutathione has been found to reduce the captan induced *in vivo* liver toxicity in the rat (Dalvi, 1988) . There are sporadic reports on the carcinogenic activity of captan. Chronic feeding studies of captan in mice have demonstrated an induction of duodnal hyperplasia and tumor formation (Stauffer Chemical Company, 1985) . Captan has been shown to be mutagenic and to induce other mutagenic effects in prokaryotes (Shirasu et al, 1976) , lower eukaryotes (Waters et al, 1980) and mammalian cells (Jingyie and Baoyeng, 1987) .

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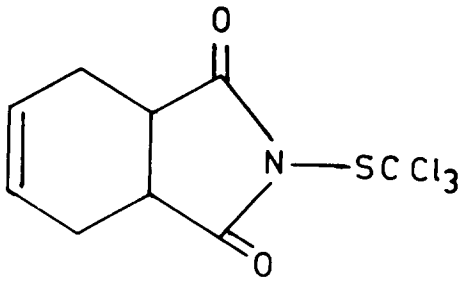

$C_9H_8Cl_3NO_2S$

Fig 1. Structure of N-trichloromethyl thiocyclohexene 1,2 dicarboximide (Captan)

In the litrature search regarding chemistry of captan in skin not much information is avilable, however its degradation in gut appears to play a major role in the metabolism. The toxic metabolite thiophosgene is produced from the trichloromethylthio moiety of the molecule in the presence of cellular thiol compounds. It is further metabolized to thiazolidine -2- thione - 4- carboxylic acid, which iss excreted in the urine of orally dosed rats; carbon dioxide is also a product of the metabolism of thiophosgene with the intermediate formation of carbonyl sulphide . Thiophosgene is also detoxified by sulphites present in the gut and is excreted in the urine of orally dosed rats to yield dithio bis(methanesulphonic acid) and its disulphide monooxide derivative (DeBaun et al, 1974).

The wide use of this fungicide and insufficient data available on its carcinogenic/cocarcinogenic potential after topical exposure has guided us to carry out the present investigation. The aim of this study is to find out whether captan can induce tumors on mouse skin after topical application in complete carcinogenic, tumor promoting and tumor initiating studies using 2 stage skin carcinogenesis protocol (Berenblum, 1975; Mehrotra et al, 1987; Shukla et al, 1988).

MATERIALS AND METHODS

Chemicals- Captan (commercial grade) was obtained from Coromandal Indag Products (P) Ltd., Madras, India. 7,12 dimethyl benzanthacene (DMBA), 3,4 benzo(a)pyrene (BaP) and 12- o - tetradecanoyl phobol- 13 -acetate (TPA) were obtained from Sigma Chemical Co., St.Louis, USA. The rest of the chemicals were of analytical grade and procured locally.

Bioassay protocol- Female, Swiss albino mice (weighing 12-15 g) were selected for these studies which included investigation of the carcinogenic, tumor promoting and tumor initiating activity of captan. The animals were kept on synthetic pellet diet and water ad libitum. Hair was clipped from a $2cm^2$ area on the interscapular skin of each mouse using electrical clippers which were not lubricated with ant oil or grease. The mice with hair cycle in the resting phase (telogen) phase of growth were selected and randomly divided into groups of 20 animals each. The test substance was administered topically on the shaved areas using the following protocols for the testing of complete carcinogenic, tumor initiating and tumor promoting potential of captan.

1. Tumor initiating activity: :To assess the tumor initiating property of captan after single (S) or multiple (M) exposure, the animals were divided into seven groups, treated as follows:

Group I	Untreated Controls
Group II (Captan(S)+ TPA)	Captan 450 m in 200 µl DMSO applied once only followed one week later by local application of 5 µg TPA in 100 µl acetone 3 times/week for 51 weeks.
Group III (Captan(M)+TPA)	Captan 450 mg /kg b.wt. dissolved in 200 µl DMSO applied 3 times\week for 3 weeks followed one week later by local application of TPA as in Group II.
Group IV (DMBA+TPA)	52 µg DMBA dissolved in 100 µl acetone applied once only, followed one week later by 5 µg TPA as in Group II.
Group V (Captan(S)+ Acetone)	Captan as in Group II followed one week later by 100 µl acetone 3 times /week for 51 weeks
Group VI (Captan(M) +Acetone)	Captan as in group III followed one week later by 100 µl acetone 3 times/week for 51 weeks.
Group VII (DMSO+TPA)	200 µl DMSO applied 3 times/week for 3 weeks followed one week later by 5 µg TPA as in Group II

2. Tumor promoting activity :For the assessment of tumor promoting activity of captan, animals were divided into 6 groups and treatment was provided topically as shown below:

Group I (Untreated controls)	No treatment.
Group II (DMBA+ Captan)	52 µg DMBA dissolved in 100 µl acetone applied once only followed one week later by captan 450 mg/kg b.wt. in 200 µl DMSO 3 times/week for 51 weeks.
Group III (DMBA + TPA)	DMBA as in Group II followed one week later by 5 µg TPA dissolved in 100 µl acetone 3 times/week for 51 weeks.
Group IV (Acetone+ Captan)	100 µl acetone once only, followed one week later by captan as in group II.
Group V (DMBA+ Acetone)	DMBA as in group II, followed one week later by 100 µl acetone 3 times/week for 51 weeks.
Group VI (DMBA+ DMSO)	DMBA as in group II followed one week later by 200 µl DMSO 3 times/week for 51 weeks.

3. Complete carcinogenic activity: For the assessment of complete carcinogenic activity of captan, animals were divided into 5 groups of 20 animals each, and treatment was provided as given below for 52 weeks.

Group I (Untreated controls)	No treatment.
Group II (BaP)	5 µg (BaP) dissolved in 100 µl acetone, 3 times/week.
Group III (Captan)	450 µg/b.wt captan dissolved in 200 ul DMSO 3 times/week.
Group IV (DMSO)	200 µl DMSO 3 times/week.
Group V (Acetone)	100 µl acetone 3 times/week.

Animals from all the 3 studies described above, were observed for gross and histopathological changes including development of tumors locally on the skin and other organs throughout the studies. Surviving animals from all experiments were sacrificed at the end of the respective study periods. Skin from the painted area (with or without tumors) was removed and fixed in 10% aqueous buffered formalin. Paraffin blocks of the tissues were prepared and 5 μ thick sections were cut, stained with haematoxylin eosin and examined histopathologically.

RESULTS AND DISCUSSION

In the group where a single dose of captan was applied poor hair growth was observed in almost all animals after day eleven of captan treatment. The first tumor was observed following 14 weeks of TPA application. At the end of 51 weeks of promotion with TPA, 3 out of 14 animals had developed tumors and the cumulative number of tumors were 4/14 (Table 1).

Table 1 Tumor initiatory activity of captan on mouse skin.

Group	Treatment	Induction of 1st tumor (in weeks)	Attainment of 100% tumorigenesis (in wks)	Total No.of animals with tumor(s) at the end point	Cumulative No. of tumors
I	Untreated controls	-	-	0/18	-
II	Captan(S)+TPA	14	-	3/14	4
III	Captan(M)+TPA	14	-	12/18	21
IV	DMBA+TPA	6	9	16/16*	90
V	Captan(S)+Acetone	-	-	0/15	-
VI	Captan(M)+Acetone	-	-	0/16	-
VII	DMSO+TPA	-	-	0/15	-

* All animals of this group were sacrificed after 10 weeks.

In the animals reciveing multiple doses of captan as the initiating agent (Group III), poor hair growth was observed in 80 % of the animals after 3 initial applications of captan. The appearence of first tumor in this group which was treated with TPA as promoter, was observed almost at the same time as noted in the group initiated with single dose of captan (Group II).

At the end of 52 weeks, 12/18 animals had developed tumor growth. The cumulative number of tumors were found to be fewer in comparison to the positive control group (Group IV) (Table 1).

On gross examination the tumors developed in the various groups described were all benign. Most of them developed as minute excrescences in the painted area which as tratment progressed, increased in size and developed as soft pinkish finger like processes whose bases were firmly attached to the skin. The latter was mobile and not fixed to the underlying tissue.Histopathological examination revealed that the tumors were benign sqamous cell pappilomas.

In the tumor promoting experiment a subcarcinogrnic dose (52 µg) of DMBA (Shukla et al, 1988) was used to initiate mouse skin which was subsequently promoted with captan thrice per week. After 2-3 applications of captan 100 % of the animals had poor hair growth. This persisted for 7-8 weeks after which a normal growth pattern was regained. After 50 days of promotion 2 animals became moriobound, developed nerve palsy, hind limb paralysis, back curving, general disability and died soon after. The remaing 18 animals survived and exhibited no difference to controls. Tumor development was not observed in any group except positive controls (Group III).

A marked loss of fur was also observed in the animals treated with captan for complete carcinogenesis, after initial few applications , but hair coat become normal after a short period despite continued captan application. After about six months of repeated application again there was a marked loss of fur on the back of treated animals and the skin appeared to be scaly and keratinized. No tumors were observed to develop in the any group treated for complete carcinogenecity, except in the BaP treated animals (positive control,Group II).

The results of these experiments indicate that topical application of captan as described above caused initiation of mouse skin in a 2-stage cancer intiation - promotion model. A single application of captan was insufficient to initiate skin for tumor development in most of the animals tested (Table 1). Multiple captan exposures led to induction of tumors in more animals (12/18), with a cumulative tumor incidence of 21 tumors during the 52 week period. Already captan has been studied in various microbial assay systems including the histidine reverse mutation system in five strains of Salmonella typhimurium. It was found to be mutagenic in these assays (Simmon et al, 1976).

While the mechanism underlying its genotoxic effects is not known, several reports have suggested that captan may interact with DNA (Stauffer Chemical Company 1981) and induce DNA repair (Ahmed et al, 1977). This DNA damaging and repair inducing character of captan was verified using the nick translation assay (Synder & Matheson, 1985). This assay measures incorporation by exogenously added E.coli DNA polymerase of radio-labelled dNTPs into cellular DNA nucleotides that have been nicked by treatment of test chemicals.

In addition to DNA strand breaks, captan is also reported to induce DNA-protein cross linking (Synder, 1982). The nature of this crosslinking is still unknown but captan has been reported to bind to histones and thus alter their ability to stabilize the DNA structure (Couch & Siegel, 1977).

From our studies it is apparent that captan does not appear to be a strong tumor initiator of the mouse skin for tumor formation. In the present set of investigations captan failed to demonstrate any promoting or complete carcinogenic activity. However, since the group size is rather smaller (20 animals/ group), hence a negative result does not allow the conclusion of no activity with much confidence. Though captan is a strong mutagen in vitro and can alter DNA in some of the cells, it, however, does not seem to be a tumor promoter or complete carcinogen in the case of mouse skin. The authors have not come across any study that had been carried out in the past to study the role of captan in multistage carcinogenesis. Some studies conducted elsewhere show that captan is an inhibitor of the hepatic microsomal enzyme system (Peeples and Dalvi, 1978). The inhibitory effect on phase I drug metabolising enzyme(s), which play a central role in activating carcinogens (Prochaska and Talalay, 1988), caused by captan, may be one of the reasons which does not permit the manifestation of tumor promoting/or carcinogenic activity by this fungicide. As captan is not metabolically activated by phase I drug metabolising enzymes, there may not be generation of electrophiles which covalently bind to the cellular targets to cause neoplasia. It has been also reported that captan is easily inactivated by cellular thiol agents. The affinity of captan to thiols may be yet another mechanism which leads to the speedy detoxification of captan by conjugation reactions. Consequently the carcinogenic and promoting propensities of captan are very weak or absent inspite of its very high mutagenic potential (Moriya et al, 1978). So the early detoxification of captan by the thiols and the lack of reactive electrophile formation may be the possible reason for the lack of tumor

promotion and complete carcinogenesis by captan when tested on mouse skin. More studies are required in this direction to establish its carcinogenic/ cocarcinogenic potential.

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