Chapter-III

Antitumour promoting activity of Indole-3-Carbinol in mouse skin carcinogenesis
Introduction

Plant materials present in the human diet contain a large number of naturally occurring compounds that may be useful to protect the body against cancer development (He et al., 2000). Recently, many candidate chemicals have been assayed to identify anticarcinogens present in our diet (Lai and Anderson, 2000; Yamada, 2000). The possible dietary anticarcinogens with promising results are indoles, isothiocyanates and monoterpenes (Wattenberg, 1990). Epidemiological evidence suggests that consumption of cruciferous vegetables is associated with a decrease incidence of cancer in the human population (Graham, 1983; Hirayama, 1986). The medicinal properties of the volatile pungent oil obtained from cruciferous plants have been observed for many centuries. Glucosinolates (a group of secondary products commonly but not exclusively present in the plants of the family Cruciferae) when chemically or enzymatically hydrolyzed give rise to a range of non-volatile indole compounds that have been implicated in the anticarcinogenic and mixed function oxidase (MFO) stimulatory activities of brassica vegetables (McDanell et al., 1988; Xu et al., 1997). Anticarcinogenic properties of cruciferous vegetables in isolated compounds have been
studied in several investigations where animals were first fed on a diet rich in cruciferous vegetables and then exposed to various carcinogens (Boyd et al, 1982; Stoewsand, 1978; Wattenberg et al, 1976). The compounds involved in the inhibition of carcinogenic activity were I3C and 3-3'-diindolylmethane (Loub et al, 1975).

Studies in various experimental models have shown that I3C can alter the metabolism of carcinogens and provide protection against chemically induced carcinogenesis (Aspy and Bjeldanes, 1983). I3C is known to inhibit mammary carcinogenesis induced by directly or indirectly acting carcinogens (Grubbs et al, 1995). It is also known to inhibit BaP-induced neoplasia of the forestomach (Wattenberg and Loub, 1978).

As we all know that repeated genetic damages are likely to be produced not only during the first step of malignancy i.e. tumour initiation but also in subsequent steps i.e. promotion and progression. Tumour promotion is generally viewed as an epigenetic event (Cerutti, 1985) but various genotoxic effects have also been detected at this stage (Ramel et al, 1986). Chromosomal and ploidy changes also appear to be important for the development of malignancy during tumour progression (Klein and Klein, 1984).

It is noteworthy that tumour promotion requires many years in humans and hence is the largest step involved in the carcinogenesis process. Therefore, a complete reversal would imply a continuous antipromoting treatment due to the reversibility of promotion and antipromotion, even some delay at this stage would result in a significant decrease in cancer incidence (Bertram et al, 1987). A prominent role in antipromotion is played by antioxidants (Cerutti, 1985; Pryor, 1986; Ramel et al, 1986; Hochstein and Atallah, 1988) such as vitamin A and retinoids, tocopherols, selenium salts, inhibitors of arachidonic
acid metabolism, inhibitors of prostaglandin synthesis, aminothiols phenols, ethoxyquin, chlorophyllin etc. (Wattenberg, 1981, 1985; Ramel et al, 1986).

Tumour promoters are well known to affect differentiation and antipromoters include differentiation inducers such as vitamin D3 analogs which enhance the survival time of mice inoculated with myeloid leukemia cells and potentiate the effects of anticancer drugs, exerting a hormone like control of either cell proliferation or cell differentiation (Kasukabe et al, 1987). There are some pharmacological agents that specifically inhibits protein kinase C (PKC), as PKC plays a central role in a variety of membrane related signal transduction events, altering the pattern of gene expression, as well as growth control and tumour promotion (Weinstein, 1987).

There are some antiphlogistic drugs such as steroidal antiinflammatory agents, having anti irritative properties, which limited the production of reactive oxygen species during inflammation (Colburn, 1980; Slaga, 1980). Inhibition of promotion also applies to physical agents such as local hyperthermia (44 °C for 30 minutes) has been shown to be a powerful antipromoter in skin carcinogenesis due to suppression of protein synthesis by heat. 2-Br-α-ergocryptine, a domamine antagonist suppressing pituitary prolactin secretion was capable of eliminating the enhancing effect of a high fat diet on mammary carcinogenesis in rats treated with DMBA (Cohen, 1986).

Tadi et al (1991) observed that organosulfur compounds, garlic and crude garlic extract are an antimutagenic and anticarcinogenic agents in aflatoxin B1 induced mutagenesis. Athar et al (1990) observed that antioxidant nordihydroguaiaretic acid (NDGA) and Dialyl sulfide shows inhibition against benzoyl peroxide mediated tumour promotion by DMBA in murine skin. Taioli et al (1997) investigated that I3C decreases lung tumour formation by the
tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by enhancing its hepatic clearance.

I3C has been found to inhibit the development of tumours in forestomach, mammary gland and liver of rodents (Wattenberg, 1990; Wattenberg and Loub, 1978; Wattenberg, 1990; Bresnick et al, 1990; Bradlow et al, 1991; Tanaka et al, 1990; Kim et al, 1994). However, dietary anticarcinogens may exhibit adverse promoting activity in certain organs such as exposure of I3C or cabbage during the post initiation (promotion stage) found to strongly enhance AFB1 induced liver tumourigenesis in the rainbow trout (Dashwood et al, 1989a, 1989b; Bailey et al, 1987) diethylnitrosamine (DEN) induced liver tumourigenesis in new born or young rats (Kim et al, 1994). Nitrosated chloroindoles and nitrosated Indole-3-acetonitrile, act as mutagenic agents with potential tumour promoting capacity (Tiedink et al, 1991). It is infact well established that a chemical act as a tumour inhibitor in one organ and as a promoter in others. So we check here the influence of I3C as an antitumour promoting agents, in this experiment.

Retrospective studies have shown a decreased risk of rectal and colon cancer following frequent consumption of cruciferous vegetables (Byers and Graham, 1984; Graham et al, 1978). Rats fed a single meal containing 25% (dry weight) cabbage showed a marked induction in MFO activity in the small intestine within 4-6 hr of ingestion (McDanell et al, 1989). I3C is a more effective enzyme inducer in comparison to indole-3-acetonitrile, 3-3′diindolylmethane and ascorbigen (McDanell et al, 1989). It is shown that ascorbic acid can react with nitrites to reduce or eliminate their availability for reaction with indoles (Wakabayashi et al, 1985). We have already checked the antigenotoxicity of I3C, antitumourigenic potential of I3C in mouse skin.
From the above experiments, we observed that I3C has preventive potential. In the present investigation the antitumour promoting activity of I3C was studied in a two stage mouse skin model of carcinogenesis using DMBA as tumour initiator and TPA as standard tumour promoter.

Materials and Methods

Chemical

DMBA, TPA and I3C were purchased from M/s Sigma Chemicals Co. USA. All other chemicals used in this study were of an analytical grade of purity and procured locally.

Animals and Treatment

Random-bred male and female Swiss albino mice of the ITRC animal colony (weighing 12-15 g) were used. They were divided into groups of 10 animals and housed in polypropylene cages under controlled temperature (22-25 °C) and humidity (65-75 °C) with a 12 h light/dark period. Animals were fed a standard synthetic pellet diet and water ad libitum. The animals were then randomly divided into different groups, each comprised of 20 animals of either sex. Hair were clipped in the dorsal region with proper care using electrical clippers that were not lubricated with any oil or grease in an area of 2 cm² in all the groups initially and every 15 days thereafter. The treatments were provided topically on the shaved dorsal skin as shown in Table 13.

Animals from all groups were examined every week throughout the experiment for gross changes locally on the skin, including loss of fur, ulcers and development of tumours. At the end of the study, all the surviving animals were sacrificed to check for tumour development in their internal organs. Tissue from the treatment site (with or without tumour
**Table 13**
Experimental Schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiation</th>
<th>Pretreatment</th>
<th>Promotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated control</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>DMBA</td>
<td>Indole-3-carbinol</td>
<td>TPA</td>
</tr>
<tr>
<td>III</td>
<td>DMBA</td>
<td>Acetone</td>
<td>TPA</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA</td>
<td>Indole-3-carbinol</td>
<td>Acetone</td>
</tr>
<tr>
<td>V</td>
<td>Acetone</td>
<td>Indole-3-carbinol</td>
<td>TPA</td>
</tr>
<tr>
<td>VI</td>
<td>Acetone</td>
<td>Acetone</td>
<td>TPA</td>
</tr>
</tbody>
</table>

*a* 52 μg of DMBA in 200 μl acetone once only, followed 1 week later by topical application of b and c.

*b* 25 μg of indole-3-carbinol in 100 μl acetone 1 hr prior to promotion twice a week for 28 weeks.

*c* 5 μg of TPA in 100 μl acetone twice a week for 28 weeks.
growth) was taken out for histopathological evaluation. Skin tumours were cleaned, washed with saline and weighed on a balance. Tumours were then fixed in 10% aqueous buffered formalin. For block preparation, after 72 hr of fixation washed the tissues in running water overnight. Then tissues were put in 70% alcohol for overnight and also gave its 2 changes in two days. After 70% alcohol, we used 90% and 100% alcohol for 2 hrs each. Then we dipped the tissues overnight into xylene.

For block preparation, melted wax was used at the temperature of 60-62 °C. Transferred the tissues into wax for atleast 3 hr. Then prepared the blocks with the help of L shaped molds. After that take out the section of 5 μm thick and transferred on Mayer's albumin coated slides.

Staining procedure start by dipping the slides into xylene for the removal of wax. Then transferred the slides into absolute alcohol, 90% and 70% alcohol for 5 minutes each. Then used running water for 2 minutes to wash the slides. After running water, stained the slides in haematoxylin for 5 minutes for nucleus staining. Then used tap water for 5 minutes. After tap water, used eosin for 2-5 minutes as a counter stain to stain different tissue fibers and cytoplasm. After eosin staining, we dehydrated the slides with the help of 70%, 90% and 100% alcohol for 5 minutes each. Then dipped the slides into xylene for 5 minutes. After xylene, mounted the slides with the help of DPX.

Statistical treatment of data was performed according to the recommendation of the IARC. In the analysis of skin tumour appearance dynamics, the Kaplan-Meier method of tumour free survival estimation was applied, considering the time to the first appearance of a skin tumour as the basic end-point (Gart et al, 1986). The data on tumour incidence were analysed by Fisher’s exact probability test between groups II and III. The average number of tumours per mouse was statistically analysed using Student’s ‘t’ test and p<0.05 was considered significant.
Results

Mortality was less in 13C treated groups. The results of the present investigation revealed that 13C inhibits DMBA-initiated and TPA-promoted mouse skin tumour formation, as evident both from a reduction in the number of animals with tumour(s) and the number of tumours per mouse (Tables 14, 15). Our findings suggest that the body weight of group II i.e. DMBA+13C+TPA increased at the end of experiment (28 weeks), in comparison to group III i.e. DMBA+Acetone+TPA (Figures 20,21) while when we compared group III with group I that is control, we found slightly increase in body weight. When compared the significance between group I to group II and group III i.e. groups DMBA+13C+TPA and DMBA+Acetone+TPA, at starting week, it was not significant (as p<0.05).

When compared the body weight of group II and III i.e. DMBA+13C+TPA and DMBA+Acetone+TPA with control group, at 4 and 8 weeks, we observed that the values was not significant to control group. But group third i.e. DMBA+Acetone+TPA showed significance when we compared it with control group at 12 weeks. At 16 weeks, there was no significance value of group II and III in comparison to group I. When we compared the significance at 20 weeks, we found that both the groups II and III i.e. DMBA+13C+TPA and DMBA+Acetone+TPA shows significant values, when compared it with control. In 24 and 28 weeks, there was a significant differences in values of group II and III i.e. DMBA+13C+TPA and DMBA+Acetone+TPA.

Our findings suggest that the number of surviving animals was high in control group i.e. I, where the number of animals at the end of experiment was 23 and 24 in both the sexes out of 25 animals (Figures 22, 23). When we compared group II i.e. DMBA+13C+TPA with group III i.e.
Table 14

Antitumour promoting potential of indole-3-carbinol in male Swiss albino mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of surviving animals with tumour at end point</th>
<th>First induction of tumour (days)</th>
<th>Attainment of 100% tumorigenesis (days)</th>
<th>% of animals with tumours at end point</th>
<th>Cumulative number of tumours</th>
<th>Average no. of tumours per mouse ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated</td>
<td>0/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>DMBA+I3C+TPA</td>
<td>9/16*</td>
<td>78</td>
<td>56*</td>
<td>63</td>
<td>7.0±1.2</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>DMBA+Acetone+TPA</td>
<td>16/16</td>
<td>47</td>
<td>100</td>
<td>146</td>
<td>9.1±1.4*</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+I3C+Acetone</td>
<td>0/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Acetone+I3C+TPA</td>
<td>0/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>Acetone+Acetone+TPA</td>
<td>0/17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Values are significantly different when compared with group III, p<0.05.
- Not detected.
Table 15

Antitumour promoting potential of indole-3-carbinol in female Swiss albino mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of surviving animals with tumour at end point</th>
<th>First induction of tumour (days)</th>
<th>Attainment of 100% tumorigenesis (days)</th>
<th>% of animals with tumours at end point</th>
<th>Cumulative number of tumours</th>
<th>Average no. of tumours per mouse ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated</td>
<td>0/19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>DMBA+I3C-TPA</td>
<td>12/17</td>
<td>63</td>
<td>71*</td>
<td>92</td>
<td>7.6±1.3</td>
<td>7.1±1.4</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+Acetone-TPA</td>
<td>17/17</td>
<td>49</td>
<td>78</td>
<td>100</td>
<td>121</td>
<td>7.1±1.4</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+I3C-Acetone</td>
<td>0/17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Acetone+I3C+TPA</td>
<td>0/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>Acetone+Acetone-TPA</td>
<td>0/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Values are significantly different when compared with group III, p<0.05.
- Not detected.
Figure 20: Showing average body weight of male Swiss albino mice at different time intervals.
Figure 21: Showing average body weight of female Swiss albino mice at different time intervals.
Antitumour promoting activity of 13C in mouse skin

DMBA+Acetone+TPA, we come to this point that the numbers of male and females was 16 and 17 in both the sexes (Figures 22, 23) at the end of experiment i.e. 28 weeks (Tables 14, 15) in group II. From these results we come to this point that 13C is slightly effective in terms to increase survival rate.

First tumour induction time was delayed in group II i.e. DMBA+I3C+TPA as tumour development was noticed after last day of 8th week while first tumour development was noticed in the early days of 7th week in group III i.e. DMBA+Acetone+TPA (Tables 14, 15). Percent age of animals with tumours was low in group II i.e. DMBA+I3C+TPA (Figures 24, 25) in both the sexes. From the Figures 24, 25, it is 9.09% and 17.3% in the 8th week while at the end of the experiment, percentages reached upto 56.2 and 70.5% in both the sexes (Tables 14, 15; Figures 24, 25) while in group III i.e. DMBA+Acetone+TPA, 9.1 and 13.6 were the percentage at the 8th week, which at the end of the experiment, increased to 100% (Tables 14, 15; Figures 24, 25). This parameter shows that 13C is effective and have inhibitory effect against TPA, tumour promoter.

The next parameter was average tumour/mouse as average number of tumour/mouse was less in group II i.e. DMBA+I3C+TPA in comparison to group III i.e. DMBA+Acetone+TPA. Number of average tumour/mouse of group II at the 8th week was 2 and 1.5 in both the sexes while it was 7 and 7.7 at the end of the experiment in both the sexes (Tables 14, 15; Figures 26, 27). The average tumour per mouse of group III at the end of 8th weeks was 3.3 and 3.5 (Figures 26, 27) in both the sexes while it increased to 9.1 and 7.1 at the end of the experiment i.e. 28 weeks (Tables 14, 15; Figures 26, 27).

Cumulative numbers of tumour was also less in group II i.e. DMBA+I3C+TPA when compared it with group III i.e. DMBA+Acetone+
Figure 22: Showing number of surviving animals in male Swiss albino mice.
Figure 23: Showing number of surviving animals in female Swiss albino mice.
Figure 24: Percentage of Swiss male albino mice developed tumours during the study period at different time intervals.
Figure 25: Percentage of Swiss female albino mice developed tumours during the study period at different time intervals.
Figure 26: Showing average tumour per mouse in different groups (male).
Figure 27: Showing average tumour per mouse in different groups (female).
TPA. As cumulative number of tumours at the 8th weeks of experiment was 4 and 6 in both the groups (Figures 28, 29) while it was 63 and 92 at the end of experiment (Tables 14, 15; Figures 28, 29) in group II i.e. DMBA+I3C+TPA. Cumulative numbers of tumours of group III i.e. DMBA+Acetone+TPA at the 8th weeks was 7 and 10 in both the sexes which was slightly high in comparison to group II at the 8th weeks while cumulative number of tumour of this group reached to 146 and 121 in both the groups at the end of this experiment that is 28 weeks (Figures 28, 29; Table 14, 15).

Skin lesions were observed in the painted area of skin on the back of mice and was defined as tumourous or nontumours. Poor hair growth, hyperkeratinization and scaly skin was observed in the non tumours lesions. Benign form of tumours was firstly observed which later transformed into malignant type. The tumours were mostly squamous cell carcinoma type. Into this form, there were several discrete squamous cell masses, which were scattered along the epidermis over a considerable area. In this disorder, the skin lesions were multiple, the histological pattern was frequently of the superficial, multicentric type. There have a greater growth potential and velocity characterized by a higher proportion of mitotic cells.

The size of the tumours in the I3C supplemented animals of group II was smaller in comparison to group III, as evident from the lesser tumour weight.

Discussion

These results suggest that I3C has an inhibitory effect in the mouse skin model of carcinogenesis. In mutagenesis, carcinogenesis and tumour promotion, reactive oxygen species (ROS) have been suggested as a causative factors in many human diseases (Balmain et al, 1992). ROS induces strand
Figure 28: Showing cumulative number of tumours in male Swiss albino mice.
Figure 29: Showing cumulative number of tumours in female Swiss albino mice.
breaks in DNA and this oxidative modifications of DNA bases leads to mutagenic and carcinogenic effects (Cerutti, 1985). When we applied TPA topically to mouse skin, it increases the release of ROS (Singh et al, 1985). ROS induce CAs and structural genetic changes resulting in alterations in gene expression with high efficiency and act as intermediate in the induction of promotion related genes. As we all know that in multistep carcinogenesis, reactive oxygen species have been shown to play a role mostly in the promotion phase. Antioxidants are reported to act as protective agents against cancer (Huang et al, 1992,1994,1997; Kozoumbo et al, 1983). Tumours observed after DMBA/TPA treatment showed necrotic keratinised squamous pearls suggesting invasive squamous cell carcinoma. On the other hand, intact basal cell layer and dysplastic lesions characterised benign papillomos in Group III treated mice. Dhawan et al (1999) observed that 4'-methyl epipodophyllotoxins, an antimitotic agent shows chemopreventive effect in DMBA/TPA induced mouse skin carcinogenesis. Wattenberg and Loub (1978) have shown that oral administration of I3C before DMBA administration inhibits the occurrence of mammary tumour in rats.

I3C, when added to the diet for 8 days prior to DMBA exposure, inhibits mammary tumour formation and also BaP-induced neoplasia of the forestomach in ICR/HA mice (Wattenberg and Loub, 1978). I3C has also been shown to inhibit chemically-induced lung tumourigenesis and has been associated with the action of chemopreventive agents on the metabolic activation and/or detoxification of carcinogens (Stoner et al, 1993). Takahashi et al (1995) showed the antimutagenic potential of I3C in Salmonella/ microsome assays. Prior to our present observation, no information was available on the antitumourigenic potential of I3C in a sequential aspect of chemically-induced skin carcinogenesis. This observation is significant since I3C has been reported to have different promoting effects in experimental
carcinogenesis (Bailey et al, 1987; Kim et al, 1997; Pence et al, 1986). Kim et al (1997) observed that I3C supplementation enhanced liver and thyroid gland neoplastic development when given during the promotion stage. Bailey et al (1987) also confirmed the tumour promoting potency of I3C in aflatoxin B1 carcinogenesis in rainbow trout. Pence et al (1986) have shown that I3C promotes 1,2-dimethyl-hydrazine-induced colon tumourigenesis in rats. In C57BL/6J mice, a statistically significant inhibition of hepatocarcinogenesis was observed in animals fed I3C and initiated with diethylnitrosamine (Oganesian et al, 1997). Exposure of I3C during the post initiation stage was found to strongly enhance 1,2-dimethyl hydrazine (DMH) induced colon tumourigenesis in rats and mice (Pence et al, 1986; Temple and El-Khatib, 1987). Morse et al (1988) observed that I3C increases liver tumour induction in F344 rats by 4-(methyl nitrosamine)-1-(3-pyridyl)-1-butane associated with augmented 7-methyl-1-guanidine adduct formation in liver DNA. McMillan et al (1986) investigated that I3C shows good potential of goitrogenicity. As its effect on the thyroid has been linked to the hydrolysis products of two glucosinolates namely 5-vinyl oxazolidine-2-thione and thiocyanate ion. The difference in the potency of I3C induced inhibition versus promotion may also depend upon the dose and frequency of exposure (Bailey et al, 1991). During oxidative stress, aldehydes are formed in biological system. These react with amino acids and DNA and form cross linkages between proteins and nucleic acids, resulting in alteration in replication transcription and leading to tumour formation (Perchellet and Perchellet, 1989). Our findings suggest that I3C also shows antitumour promoting potential as it is an antioxidant and block the ultimate carcinogenic electrophiles by forming innocuous products in a nucleophilic chemical reaction.