Chapter-IV

Effect of Indole-3-Carbinol on Ehrlich ascites tumour bearing Swiss albino mice
Introduction

Cancer cells are known to divide where normal cells don't they invade, metastasize and kill the host of origin. Cancer is inheritable at the cellular level and the cells of cancer contain multiple mutations (Leob and Leob, 2000). One of the important thing about cancer cells is that these are genetically instable. This is manifested both at the single nucleotide level, resulting in point mutation or at the chromosomal levels resulting in translocations, deletions, amplifications and whole chromosomal aneuploidy (Leob and Leob, 2000). This genetic instability, we observed in our first experiment as when cyclophosphamide (CP) which is an antineoplastic agent was given alone and in combination with I3C to Swiss albino mice. The group treated with CP only showed higher rate of CAs in comparison to I3C treated group. In our 2nd and 3rd chapter of this dissertation also we have seen the effect of anticarcinogenesis on mouse skin as in the form of complete carcinogenesis and antitumour promoting activity of I3C but the tumours were of solid forms where we used DMBA as tumour initiator and TPA as tumour promoter. As there was no study of I3C on Ehrlich ascites tumour transplant mice, that's why we chose this.
As cancer expands they encounter a series of restrictive blockades that limits further growth. These limitations to growth might include interference by surrounding tissue, reduced nutrition, reduced oxygen levels need for growth factors, inadequate blood supply, etc. Each of these blockades might be overcome by mutations. There are certain dietary constituents which played important role in DNA damaging. Ames et al (1995) observed that many foods contain different natural chemicals that also damage DNA and produce alterations. Consumption of natural DNA damaging chemicals from food is much greater than exposure to DNA damaging chemicals produced by industry (Ames et al, 1990).

Shevchuk et al (1998) observed that (2)-t-butyl-4-hydroxyanisole (BHA) a widely used food antioxidant, inhibited the haematoporphyrin derivate (HpD) based photosensitizied injury of Ehrlich ascites tumour cells. Orosz et al (1999) investigated that a new semisynthetic antitumour bis-indole compound, KAR-2[3'-((beta-chloroethyl)-2', 4'-dioxo-3,5'-spiro oxazolidino-4-deacetoxyvinblastine] shows prolonged life span of Ehrlich ascites tumour transplant mice. Yamamoto and Naraparaju (1998) observed that vitamin D3 binding protein has a potent adjuvant activity for immunization in transplanted Ehrlich ascites mice. Saris et al (1998) observed that EA cells inhibited the permeability transition and phospholipase A2 activity by magnesium thats why EA cells have a high Ca2+ threshold. Verma et al (1999) investigated the potential of ascites fluid absorbed in vitro over non-viable Staphylococcus aureus Cowan I (SAC) containing Protein A (PA) in Ehrlich's ascites tumour in mouse. There was a significant decrease in body weight as well as significant reduction in viability of ascites tumour cells in peritoneal cavity. Shenoy et al (1999) observed that antitumour efficacy of injectable centchroman as it showed a significant increase in host life span. Compared to free drug, this increases the antitumour efficacy against Ehrlich ascites carcinoma. Butyric acid derived
from colonic fermentation of dietary fibre not only helps in inhibiting tumour growth but also acts as an efficient anti inflammatory agent (Salimath \textit{et al}, 1999). The drug Dicyclopenadienyldichlorotitanium (IV) (DDCT) protects the myelosuppression induced by the tumour (Valadares \textit{et al}, 1998). Chaudhuri and Chatterjee (1998) investigated that a drug resistant cell line was developed by repeated exposure of Ehrlich ascites (EAC) carcinoma cells to Doxorubicin (DOX) \textit{in vivo} in male mice. This resistance appeared to be due to the presence of a higher level of reduced glutathione (GSH), to glutathione S-transferase (GST) in EAC/DOX cells than in drug sensitive EAC/S cells. Ghosh \textit{et al} (1999) investigated that boron compound of guanidine biboric acid adduct (GH) and guanidine chloride showed an antitumourigenic effect against EAC in mice by increasing the survival time and also showing minimum toxic effects when used haematological parameters.

Our first set of investigation carried out to find the cytotoxicity of test compound I3C through Ehrlich ascites tumour transplant mice. Ekmekcioglu \textit{et al} (1999) investigated that fresh oranges, packaged orange juice, mineral water, drip coffee, camomile tea, black tea, green tea, cola and energy drinks exerts toxic and growth/cell proliferation effects on cells. Cells showed higher mitochondrial metabolic activities. Futami \textit{et al} (1999) observed that RNase inhibitor is considered to play a protective role against the endocytotic RNase from outside of cells. This RNase fibroblast growth factor (FGF) fused protein effectively inhibited the growth of mouse melanoma cell line B16/BL6 with high levels of cell surface FGF receptor. Thomas \textit{et al} (1999) observed that sulphur containing substrate (tyrosine) analogs, N-acetyl-4-S-cysteaminyl phenol (NAcCAP) and N-propionyl-4-S-cysteaminyl phenol (NPrCAP) have potent anti melanoma activity in mice. Ethylene diamine tetra acetic acid (EDTA), a chelator form of metal ions are used in modern medicine and pharmacy has cytotoxic side effects. The side effects of EDTA included
breast milk cell loss, disruption of milk fat globule membrane and subsequent release of membrane bound protein; free fatty acids and reduction in pH (Ogundele, 1999). Yang et al (1999) observed that cAMP activation and tyrosine kinase signalling are involved in the mitogenic response of melanocytes which increased the susceptibility of these cells to cytotoxic effect of 4-tertiary butyl phenol (4-TBP).

Angiogenesis is best studied in the growth of malignant tumours, since cancer may be regarded as the most important angiogenesis dependent diseases in terms of social and economic aspects (Obermair et al, 1999). Desai and Libutti (1999) investigated that angiogenesis is the ability of pre existing vasculature to send out capillary sprouts leading to the formation of new vasculature. It is now well known that progression of solid tumours is intrinsically dependent an angiogenesis for growth of the primary tumours and metastatic lesions. Iseki et al (1999) observed that both 1 alpha-hydroxy vitamin D3 [1 alpha(OH) D3] and 1.25-dihydroxy vitamin D3 [1.25 (OH) 2D3] inhibits development of colon carcinogenesis and angiogenesis. Purine analog 6-methylmercaptopurine (6-MMPR) specifically inhibits both early and the late phases of the angiogenesis process in in vitro and in vivo also (Presta et al, 1999). Fontamini et al (1999) observed that vascular endothelial growth factor (VEGF) protein expressed association with neovascularization both in human cancers and in various types of pre invasive lesions. Bauer et al (1998) observed that thalidomide act as inhibitor of angiogenesis of rabbit cornea micropocket model only after their metabolic activation. Selective cyclooxygenase-2(cox-2) inhibits TXA₂ production, endothelial migration and fibroblast growth factor induced corneal angiogenesis (Daniel et al, 1999).

The present set of investigation was carried out to find the antitumourigenic effect of I3C in Swiss albino mice bearing EA tumour. A tumour must
continuously stimulate the growth of new capillary blood vessels, for the
tumour itself to grow. The new blood vessels embedded in a tumour provide
a gateway for tumour cells to enter the circulation and to metastasize the
distant sites such as liver, lung or bone.

Materials and Methods

Chemicals

I3C was purchased from M/s Sigma Chemicals Co. USA. Trypan blue
was of analytical grade and obtained through commercial sources.

Animals and Treatment

Random bred Swiss albino mice (male of 20-22 gm body weight) were
obtained from Industrial Toxicology Research Centre (ITRC) animal colony.
They were kept in groups of 10 animals per group in polypropylene cage,
under controlled temperature (22-25°C) and humidity (60-70%) with 12/12 h
light/dark period. Animals were fed a standard solid pellet diet and water ad
libitum. Ehrlich ascites tumour was taken from the Ehrlich ascites tumour
bearing mice of ITRC animal colony of Gheru campus.

The first set of experiment was observed for increased life span (ILS).
Here, EA cells were maintained (Figures 30,31) by cells which was extracted
from peritoneal cavity and washed with saline and suspended in phosphate
buffer saline (pH 7.0).

For this, animals were divided into 6 groups of 10 animals each. I3C (5
mg/kg body weight) was given orally prior to inoculation of EA tumour cells
in 3 groups. Then, in all the three groups 1x10^6, 2.5x10^6 and 5x10^6 cells/ml
were inoculated i.p. after 1 week of I3C treatment. In untreated groups, also
1,2.5 and 5 million cells were inoculated.
Figure 30: Ehrlich ascites tumour transplant mouse (initial stage)

Figure 31: Ehrlich ascites tumour transplant mouse (advance stage)
The percentage of increased life span was calculated as described by Reibscheid *et al* (1964).

\[
\% \text{ ILS} = \frac{T - C}{C} \times 100
\]

*where:*

- **ILS** = Increased life span
- **T** = Average life span of treated group
- **C** = Average life span of control group

The value more than 25% is taken as significant increase in ILS.

In the second set of investigation, 0.5 ml EA cells were extracted from the peritoneal cavity and washed with normal saline and the volume was made up 5 ml by adding normal saline. EA cells of 0.5 ml was dispersed in tube and 13C was added at different concentration (2-20 nmoles per plate and 5-50 nmoles per plate). Cells were incubated at 37°C for 3 hours. After incubation they were stained with 1% trypan blue. Cell counting was done using haemocytometer under a light microscope. Atleast 200 cells were counted.

\[
\text{Cytotoxicity} = \frac{\text{Number of dead cell}}{\text{Total number of cell counted}} \times 100
\]

**Results**

The results of the present investigation show that I3C was effective in terms of increased life span of tumour bearing animals. There was an increase in the life span of 1 million, 1 million +I3C treated group as its life span increased to 60.7% (Figure 32) in comparison to 2.5 million + I3C and 5 million cells + I3C cells. Values of group I and group II are significant as group I's increased life span was 60.7 and group II was 30.6% (Figure 32).
Figure 32: Showing increased life span of Swiss albino mouse.
the second set of experiment, I3C showed cytotoxicity by increasing its concentration (Figures 33, 34). For studying the antitumour activity of test compounds, the increase in life span and cytotoxicity in Ehrlich's ascites tumour model, are well established parameters (Mitsui et al., 1995; Nishida et al., 1994). There was a slight increase in the blood vessels in the form of angiogenesis when we compared in group II and III (Table 16) where we inoculated 1 million cells and 1 million + I3C to group I that is control. When compared the group I i.e. control with group II and III that is 2.5 million cells and 2.5 million + I3C we found that there was also slight increase in the process of angiogenesis (Table 16) in group III. But when we observed the results of group I to group II and III i.e. 5 million cells and 5 million cells + I3C (Table 16) we found no marked changes in the blood vessels. In group I where 1 million cells were inoculated in untreated group in comparison to treated group i.e. I3C + 1 million cells. Life span of both the groups were same (Figure 35). In second group where we inoculated 2.5 million cells in untreated group in comparison to I3C + 2.5 million cells, inoculated in treated group i.e. II, life span of untreated group was slightly high in comparison to treated group (Figure 36). While in case of third group, when inoculated 5 million cells in untreated group and I3C + 5 million cells in treated group life span of both the group were same (Figure 37). So from the above result we can say that I3C does show some effect in terms of increased life span of mice.

**Discussion**

It is evident that tumourigenesis is in many cases a multistep process that begins with the abrogation of normal control on cell oncogens and/or the loss of tumour suppressor genes (Bishop, 1991). Typically, this process yields malignant tumour cells with a number of aberrant characteristics including
Figure 33: Cytotoxicity of 13C at low concentration
Effect of I3C on Ehrlich ascites tumour

Figure 34: Cytotoxicity of I3C at high concentration
Table 16
Effect of I3C on peritoneal vasculature at different concentration.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Effect of I3C on peritoneal vasculature</th>
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<tr>
<td><strong>Table 16a</strong></td>
<td></td>
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<tr>
<td>I (Control)</td>
<td>-</td>
</tr>
<tr>
<td>II (1 million cells)</td>
<td>+</td>
</tr>
<tr>
<td>III (1 million cells + I3C)</td>
<td>++</td>
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<tr>
<td><strong>Table 16b</strong></td>
<td></td>
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<tr>
<td>I (Control)</td>
<td>-</td>
</tr>
<tr>
<td>II (2.5 million cells)</td>
<td>+</td>
</tr>
<tr>
<td>III (2.5 million cells + I3C)</td>
<td>++</td>
</tr>
<tr>
<td><strong>Table 16c</strong></td>
<td></td>
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<tr>
<td>I (Control)</td>
<td>-</td>
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<tr>
<td>II (5 million cells)</td>
<td>-</td>
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<tr>
<td>III (5 million cells + I3C)</td>
<td>-</td>
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(-) Not effective
(+ ) Slightly effective
(++) Highly effective
Effect of 13C on Ehrlich ascites tumour

Figure 35: Inoculation of $1 \times 10^6$ EA cells.
Treated- shows the inoculation of 13C$1 \times 10^6$ cells.
Untreated- shows the inoculation of $1 \times 10^6$ cells.
Figure 36: Inoculation of $2.5 \times 10^6$ EA cells.  
Treated- shows the inoculation of $13C \times 2.5 \times 10^6$ cells.  
Untreated- shows the inoculation of $2.5 \times 10^6$ cells.
Figure 37: Inoculation of $5 \times 10^6$ EA cells.
Treated- shows the inoculation of $13 \times 5 \times 10^6$ cells.
Untreated- shows the inoculation of $5 \times 10^6$ cells.
genomic disarray, rapid proliferation, invasive and migratory capabilities. It is recognised that a crucial step in the development of solid tumour is the acquisition of the capacity to induce new blood vessel growth (Folkman, 1990).

Angiogenesis plays an important role in tumour cell invasiveness and metastases as it provides sufficient blood supply to cancer cells for the proliferation. But there are some inhibitors which shows characteristic antitumourigenic effect. Our findings suggests that I3C, is also act as inhibitor in the process of angiogenesis. Qian et al (1999) investigated the effect of angiogenic inhibitor TNP-470 on human squamas cell nasopharayngeal carcinoma (NPC) and found effective against this carcinoma. Bauer et al (1998) found that a metabolite of thaidomid is responsible for its angiogenic effect and this metabolite can be formed in both human and rabbit but not in rodents. Angiotensin II (AII) is receptor angiotensin converting enzyme inhibitors (ACEI) and AT-1 receptor antagonists (ATIRA) decreases the process of angiogenesis and cellular proliferation and favours cellular differentiation which showed the protective effect of ACEI against cancer (Fournier et al, 1999). Osawa et al (1999) found that AGM-1470, an angiogenesis inhibitor inhibited the growth and malignant progression of N-butyl-N-C4-hydroxybutyl) nitrosamine (BBN) induced bladder tumours in rats.

The sulfonic acid polymers, poly (2-acrylamido-2-methyl-1-propane sulfonic acid (PAMPS), poly (4-styrenesulfonic acid (PSS) and poly anetholesulfonic acid (PAS) have been proved to be highly potent inhibitors of angiogenesis in the chick chorioallantoic membrane (CAM) assay (Leikens et al, 1977). Nguyen et al (1993) investigated that pentosan polysulfate (PPS) is a highly negatively charged polysaccharide and is a potent inhibitor of tumour associated angiogenesis and is an effective agent for the prevention
and/or suppression of prostate cancer growth. Rose *et al* (1999) observed that dietary glycine prevents tumour growth *in vivo* by inhibiting endothelial cell proliferation.

There are some agents which are positive for angiogenesis such as matrix metalloprotein which played an important role in tumour angiogenesis and growth (Drummond *et al*, 1999). The urokinase type plasminogen activator (UPA) system plays an important role in tumour cell invassiveness, metastates and angiogenesis.

Cytotoxicity can be delayed or inhibited by some inhibitors which plays an important role in this process. El-Bahay *et al* (1999) found that tumour necrosis factor alpha-amanitin causes delayed cytotoxicity by rapid inhibition of RNA and protein synthesis. Cytoprotection and iron modilization effect by a new dihydroxamate chelator 1,1 bis[11-N-hydroxy]-2,5, 11-triaza-1,6,10-trioxododecanyl] ethane or KD was observed by Rakba *et al* (1999). KD is effective comparable to desferrioxamine (DFO) in protecting rat hepatocytes. Renwick *et al* (1999) observed that I3C or I3A showed marked reduction of cytotoxicity in liver slices. Hermanns *et al* (1999) in their research found that antiseptics are beneficial in bacterial contaminated wound healing and also in the treatment of chronic cutaneous ulcers. Chlorophyll and its derivatives pheophytin, pyropheophytin and pheophorbide, found in vegetables have a significant role in cancer prevention and all these are antimutagenic against 3-methyl cholantherene (3-MC).

Metallothionein (MT) a metal regulatory protein of zinc shows a protective effect against diethyl-dithiocarbamate cytotoxicity on rat hepatocytes *in vitro* (Wilson and Tromsbotta, 1999). Traore *et al* (1999) observed that Okadic acid, a marine toxic has a cytotoxic potential and it promotes tumours in skin but it is a specific potent inhibitor of protein synthesis and also inhibits phosphatases A₁ and A₂ *in vitro*. Ashizawa *et al* (1999) found that KF 22678,
novel thioether derivative of leinamycin with the 1-oxo-1,2-dithiolane-3-one showed antitumour activity against human carcinoma xenografts (lung, colon, ovary and prostate). Capsaisin a natural product of capsicum species showed cytotoxicity and genotoxicity in human neuroblastoma cells (Richeux et al., 1999).

From the earlier studies we come to this conclusion that I3C is effective and reduced cytotoxicity of liver slices, but it is not much effective when we increased the concentration of I3C.

Our third experiment was to observe the effect of ISC on Ehrlich ascites tumour cells. From the results, we observed that I3C was slightly effective in terms of increased life span but when the concentration of EA cells was high in the last group then I3C was not effective.

But there are some drugs which showed increased life span of Ehrlich ascites tumour bearing mice. Goel et al (1998) investigated that P. hexandrum, a herb, occurring at Himalays, shows antitumour activity in strains of mice carrying solid tumours, developed by transplanting Ehrlich ascites tumour (EAT). Ixora coccinea L (Rubiaceae) flowers showed antitumour activity in intraperitoneally transplanted Dalton's lymphoma ascitic (DLA) and solid tumours and also Ehrlich ascites carcinoma tumours in mice (EACT). The active fraction of I. coccinea flowers increases the life span of DLA and EACT bearing mice as it inhibited the tritiated thymidine incorporation in cellular DNA (Latha and Panikkar, 1998). Mylonaki et al (1998) found that carboplatin (cPt) reduces the ascitic volume of EAT bearing mice.

Dwarakanath et al (1999) observed that two deoxy-D-glucose (2-DG), an inhibitor of glucose transport and glycolysis inhibited the repair of radiation damage in cancer cells by reducing the flow of metabolic pathways.
The available literature suggests that our findings are in accordance with other studies. As I3C act as inhibitor but the effectiveness was not high. We can conclude that I3C is effective against CP induced genotoxicity and also positive against two stage mouse skin model. So our findings suggest that I3C is a good candidate of cruciferous vegetables as it is beneficial for human beings.