CHAPTER 6

ANTI-CARCINOGENIC POTENTIAL OF

CAROTENOID MESO-ZEAXANTHIN

AND ITS POSSIBLE MECHANISM OF ACTION
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6.9. DISCUSSION
6.1. INTRODUCTION

Human beings are continuously exposed to varying amounts of chemicals that have been shown to have carcinogenic or mutagenic properties in experimental systems. Exposure can occur exogenously when these agents are present in food, air or water, and also endogenously when they are products of metabolism or pathophysiologic states such as inflammation. It is estimated that exposure to environmental chemical carcinogens contribute significantly to the causation of a majority of human cancers (Belpomme et al., 2007). Despite significant advances in medicine, liver cancer, predominantly hepatocellular carcinoma remains a major cause of death worldwide. As limited treatment options are currently available to patients with liver cancer, new preventive and therapeutic approaches are to be adopted to combat this disease. Several naturally occurring dietary and non-dietary phytochemicals have been shown to have enormous potential in the prevention and treatment of several cancers, especially those of the gastrointestinal tract (Surh, 2003; Zhang et al., 2012).

N-nitroso diethyl amine (NDEA) is a N-nitroso compound found to produce hepatotoxicity in experimental animals after repeated administration (Anna et al., 1994). NDEA is present in variety of food stuffs like cheese, soybeans, smoked, salted and dried fish, cured meat, alcoholic beverages etc (Froment et al., 1994). NDEA becomes metabolically active by the action of cytochrome P450 enzymes to produce reactive electrophiles, which increases oxidative stress leading to cellular damage, mutagenicity and carcinogenicity (Telliez et al., 1995). Oxidative stress is considered as critical mechanism contributing to NDEA induced hepatotoxicity, and the use of anti-oxidants can reduce the liver damage.

Sarcoma is a cancer that arises from transformed cells of mesenchymal origin. Solid tumours of connective tissues such as muscle, bone, cartilage and fibrous tissues are, by definition, considered sarcomas. Sarcomas affect people of all ages. Approximately 50% of bone sarcomas and 20% of soft tissue sarcomas are diagnosed in people under the age of 35 (Darling, 2007). Some sarcomas, such as chondrosarcoma, leiomyosarcoma and gastrointestinal stromal tumour (GIST), are more common in adults than in children. Most high-grade bone sarcomas,
including Ewing's sarcoma and osteosarcoma, are much more common in children and young adults.

Chemical carcinogenesis in murine and in human skin is a multi step process including initiation, promotion and progression (Aggarwal and Mukhtar, 1991; Friedrich et al., 1995). In mouse skin, a single topical application of 7, 12-dimethylbenz [a] anthracene (DMBA) effects initiation while promotion is accomplished by repeated topical applications of a promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Slaga et al., 1996; Digiovanni, 1992). Many human cancers develop through a multi-step process as in the case of two-stage papillomagenesis in mouse skin. So agents intervene in either initiation or promotion phases of papillomagenesis provide better approaches to human cancer prevention, intervention and therapy.

meso-Zeaxanthin (MZ) is a carotenoid present in the fovea centralis of retina. Previous studies showed that MZ has antioxidant, anti-inflammatory, anti-mutagenic, cytotoxic and anti-tumour activities. In this study, we describe the effect of MZ against 3-methylcholanthrene (3-MC) induced sarcoma, NDEA induced hepatocellular carcinoma, and DMBA and croton oil induced two-stage skin papilloma formation. In order to determine the mechanism of anti-carcinogenic action of MZ, the effect of MZ administration on various CYP450 isoenzymes and phase II enzymes were studied.
6.2. MATERIALS AND METHODS

6.2.1. Chemicals

Nitroso diethyl amine (NDEA), 3-Methylcholanthrene (3-MC), 7,12-dimethylbenz[a]anthracene (DMBA), resorufin, 7-ethoxy resorufin (ER), 7-pentoxyresorufin (PR), 7-Methoxy resorufin (MR) were purchased from Sigma-Aldrich Inc. (St. Louis, Missouri, USA). Phenobarbitone (Gardenal R 60, Batch No.B03007) was bought from Nicholas-Piramel India Ltd, Gujarat. Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

6.2.2. Animals

Male Swiss albino mice (6-8 week old, 25 ± 3 g.wt) and male Wistar rats (5-6 week old, 180 ± 20 g.wt) were used in the study.

6.2.3. Anti-carcinogenic Activity of MZ against NDEA Induced Hepato Cellular Carcinoma

Thirty male Wistar rats were divided into 5 groups (6 animals/group)

Group I: Normal
Group II: NDEA alone treated (NDEA Control)
Group III: NDEA+ Sunflower oil (Vehicle Control)
Group IV: NDEA+ MZ (50 mg/kg b.wt)
Group V: NDEA+ MZ (250 mg/kg b.wt)

NDEA (0.02%) was freshly prepared in distilled water every day. Animals in group II-V were fed with NDEA (2.5 ml/rat/dose) orally 5 days in week for 20 consecutive weeks. This dosage was found to produce liver cancer in rats within 20 weeks (Jose, Joy and Kuttan, 1999). Various concentrations of MZ (50 and 250 mg/kg b.wt) were administered orally 5 days in a week for 20 consecutive weeks. Administration of NDEA and drug were stopped at 20th week and animals were kept under observation for another 9 weeks. At the end of 29th week all the animals were sacrificed. Gross necropsy of animals was made to see any visible morphological changes of the organs. Blood was collected from each animal through heart puncture and was collected in heparinised tubes to separate the serum.
6.2.3.1. Parameters Assessed

- **Survival Rate, Morphology and Weight of the Liver**
  
  Mortality of animals in each group was monitored every day. Livers from each animal were excised after sacrifice, washed in ice-cold saline (0.9%) and observed for tumour nodules and other morphological abnormalities. Weight of each liver was recorded and was expressed as liver weight/100 g b.wt.

- **Enzyme Analysis in the Liver and Blood**
  
  Serum separated was used to determine the activities of alkaline phosphatase (ALP) (Kind and King, 1954), total bilirubin (Malloy and Eyelyn, 1937), γ-glutamyl transpeptidase (Schumann et al., 2002), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) (Reitman and Frankel, 1957) using commercially available kits (Span Diagnostics, Gujarat, India). A 25% homogenate of the liver tissue was prepared in cold Tris-HCl buffer (0.1M, pH 7.4) and was used for the estimation of GSH and liver enzymes (Chapter 2, section 2.2.11.2 to 2.2.11.5). GOT, GPT, ALP, total bilirubin and γ-GT level in the liver homogenate was determined using commercially available kits. Protein content was determined by the method of Lowry et al., 1951.

- **Histopathological Analysis**
  
  A small portion of liver was fixed in 10% formalin for histopathological analysis.

6.2.4. Anti-carcinogenic Activity of MZ on 3-MC Induced Sarcoma

Sixty male Swiss albino mice were randomly divided into four groups (15 animals/group). Hair from the dorsal side of the animals was removed 24 hours before the experiment. A single dose of 3-MC (200 µg/animal/dose) in 0.1 ml of dimethyl sulphoxide (DMSO) was administered subcutaneously (s.c) on the dorsal surface of each animal to induce sarcoma (Joy et al., 1999).

- Group I: 3-MC alone (3-MC control)
- Group II: 3-MC+ Sunflower oil (vehicle control)
- Group III: 3-MC +MZ (50 mg/kg b.wt)
- Group IV: 3-MC +MZ (250 mg/kg b.wt)
The treatment with MZ was started (oral gavage) 24 hrs after the injection of 3-MC and was continued for 6 days in a week for 20 consecutive weeks. The animals were observed for 30 weeks for the onset of sarcoma as well as their survival.

6.2.5. Anti-carcinogenic Activity of MZ on DMBA and Croton Oil Induced Two-stage Skin Papillomagenesis

A total of eighty male Swiss albino mice were used for the study. They were divided into ten groups of 8 animals/group. Aggressive males were removed and kept separately. The dorsal skin of the animals was shaven 3 days before the commencement of the experiment and only those animals in the resting phase of hair cycle were chosen for the study. Single dose of DMBA (470 nano mol/mouse in 200 µl acetone, topical) was painted in the shaven area (Reddy and Failkow, 1983). Here DMBA was used as an initiating agent and 1% croton oil was used as a promoting agent. Croton oil was applied two weeks after DMBA application. Animals were divided into different groups.

Group I: DMBA alone (initiation only)
Group II: croton oil alone (200µl/mouse, topical, twice weekly for 8 weeks)
Group III: DMBA + croton oil (200µl croton oil/mouse, topical, twice weekly for 8 weeks)-Control.
Group IV: DMBA+ croton oil + sunflower oil (200µl sunflower oil/mouse, topical, 30 minutes before each croton oil application, twice weekly for 8 weeks) (Vehicle control).
Group V: DMBA+ croton oil + MZ (0.1% MZ/mouse, topically for 10 continuous days prior to the application of DMBA) (Prior to initiation)
Group VI: DMBA+ croton oil + MZ (0.2% MZ/mouse, topically for 10 continuous days prior to the application of DMBA) (Prior to initiation)
Group VII: DMBA+ croton oil + MZ (1% MZ/mouse, topically for 10 continuous days prior to the application of DMBA) (Prior to initiation)
Group VIII: DMBA+ croton oil + MZ (0.1% MZ/mouse, topical, 30 minutes before each croton oil application, twice weekly for 8 weeks) (Prior to promotion)
Group IX: DMBA+ croton oil + MZ (0.2% MZ/mouse, topical, 30 minutes before each croton oil application, twice weekly for 8 weeks) (Prior to promotion)

Group X: DMBA+ croton oil + MZ (1% MZ/mouse, topical, 30 minutes before each croton oil application, twice weekly for 8 weeks) (Prior to promotion)

Skin tumour formations were recorded weekly and the tumours greater than 1mm diameter were included in the cumulative total if they persisted for two weeks or more. Delays in the onset of tumours in various groups were recorded.

6.2.6. Determination of the Effect of MZ on Phase I Carcinogen Metabolising Enzymes in vitro

6.2.6.1. Induction of Cytochrome P450 Enzymes

Rats were administered with phenobarbitone (which can induce microsomal P450 enzymes) continuously for 4 days (60 mg/kg b.wt, intra peritonially, once daily). They were sacrificed 24 hours after the last dose of phenobarbitone. The livers of all the animals were excised quickly and washed thoroughly in ice-cold saline and kept at -70°C. Liver homogenate (25%) was prepared in cold phosphate buffer (pH 7.4, 0.1M). Homogenate was initially centrifuged at 14000 rpm for 20 minutes in a cold centrifuge (Remi) and supernatant was then further centrifuged at 10,500 rpm for 1 hour in an ultracentrifuge (Sorvall) and microsomes obtained were washed and resuspended in cold phosphate buffer (pH 7.4, 0.1 M) and used for the analysis of various CYP450 enzymes.

6.2.6.2. Effect of MZ on Different Isoforms of Microsomal Cytochrome P450 Enzymes In vitro

To study the effect of MZ on CYP450 isoenzymes in vitro, various concentrations of MZ (5-20 µg/ml) were incubated with microsomal fraction. The effect of MZ on the dealkylation of methoxy resorufin by 7-methoxyresorufin-O-demethylase (MROD)-CYP1A2, pentoxy resorufin by 7-pentoxyresorufin–O-depentylase (PROD)-CYP2B1/2 and ethoxy resorufin by 7-ethoxyresorufin-O-deethylase (EROD)- CYP1A1 were studied (Pohl, Fouts, 1980; Nerurkar et al., 1993). Assay mixture contained sodium phosphate buffer (0.1M, PH 7.4), 6.25 mM
MgSO₄, 60 µM EDTA, 5 µM ER, MR or PR, 100 µg microsomal protein, 100 µM NADPH and various concentrations of MZ in a final volume of 1ml. The reaction time was 5 minutes with a pre-incubation period of 5min without the addition of NADPH at 37°C. The reaction was stopped by the addition of 2 ml of chilled methanol. The precipitated protein was centrifuged down and supernatant was used for the estimation of enzyme activity using a fluorescent spectrophotometer (Hitachi F-2500) at the excitation wavelength of 550 nm and the emission wavelength of 585 nm. Blanks were set without any NADPH. The percentage inhibition was calculated by the following formula.

\[ \text{C-T/C} \times 100 \]

Where C is the optical density of control without MZ, T is the optical density with MZ.

6.2.6.2.1. Inhibition of Aniline Hydroxylase Activity (An Indicator of CYP 2E1 Activity)

Inhibition of aniline hydroxylase activity (an indicator of CYP 2E1 activity) was measured by the method of Mazel (Mazel, 1971).

**Principle**

Aniline hydroxylase catalyse the hydroxylation of aniline to p-aminophenol in the presence of NADPH. The activity of aniline hydroxylase was determined by measuring the quantity of p-aminophenol formed. P-aminophenol formed is allowed to react with phenol in an alkaline medium to form a blue coloured product, which can be measured at 630 nm.

**Protocol**

The reaction mixture (1.5 ml) containing microsomal fraction (1.5 mg protein), phosphate buffer (150 mM, PH 7.4), MgCl₂ (5 mM), aniline (32 mM in ethanol) and various concentrations of MZ was incubated at 37°C for 5 minutes. NADPH (0.33 mM in buffer) was added to the mixture and the incubation was continued for 2 hours at 37°C. After the incubation, 500 µl of 20% TCA was added to the mixture and centrifuged at 3000 rpm for 10 minutes. 1.5 ml of supernatant was mixed with 750 µl of 10% Na₂CO₃ and 1.5 ml of phenol (2% in 0.2M NaOH). Then
the mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 630 nm against the buffer and the percentage of inhibition was calculated.

**6.2.6.2.2. Inhibition of Aminopyrene-N-demethylase Activity (An Indicator of CYP 1A, 2A, 2B, 2D and 3A Activity)**

The method of Mazel (Nash, 1953; Mazel, 1971) was adopted to measure inhibition of aminopyrene-N-demethylase activity (an indicator of CYP 1A, 2A, 2B, 2D and 3A activity).

**Principle**

Aminopyrene is dealkylated by microsomal enzyme aminopyrene-N-demethylase to form 4-amino antipyrene and formaldehyde. Formaldehyde so formed was measured by condensation with Nash reagent.

**Protocol**

The total volume of the reaction mixture was 1.5ml. The reaction mixture containing microsomal fraction (1.5 mg protein), phosphate buffer (150 mM, pH 7.4), MgCl₂ (5 mM), aminopyrene (32 mM), semicarbazole hydrochloride (120 mM) and various concentrations of MZ was incubated at 37°C for 5 minutes. NADPH (0.33 mM in buffer) was added to the mixture and incubated again for 2 hours at 37°C. After incubation, the reaction was stopped by the addition of a mixture of 10% ZnSO₄ (500 µl) and saturated Ba(OH)₂ solution (500 µl). The mixture was centrifuged at 3000rpm for 10 minutes. The supernatant was mixed with 500 µl of Nash reagent [Nash reagent is a mixture of ammonium acetate (30 g) and acetyl acetone (400 µl) in a total volume of 100 ml distilled water]. The tubes were then placed in waterbath at 60°C for 30 minutes for colour development and absorbance was measured at 412 nm against distilled water. The percentage of inhibition was calculated.

**6.2.6.3. Effect of MZ on Phase I Carcinogen Metabolising Enzymes In vivo**

Thirty rats were randomly divided into five groups (6 animals/group) as follows

- **Group I**: Untreated (Normal)
- **Group II**: Phenobarbitone alone treated (PB control)
- **Group III**: Sunflower oil + phenobarbitone (vehicle control, VC)
- **Group IV**: Phenobarbitone+ MZ (50 mg/kg b.wt)
Group V: Phenobarbitone+ MZ (250 mg/kg b.wt)

MZ was administered orally once daily for 15 days. Administration of phenobarbitone (60 mg/kg b. wt., intra peritonially, once daily) was started on 12th day and continued for 4 days. On the 16th day 1 hour after MZ administration animals were sacrificed and the liver was excised out. The liver microsomal fraction from MZ treated and untreated animals were prepared as given above and were used to determine the activities of EROD, MROD and PROD. Calibration curves were constructed by determining the fluorescence of known amounts of the authentic resorufin. Concentration of protein in each sample is measured by the method of Lowry et al. Results were expressed as nano mols of resorufin formed/min/mg protein for in vivo studies.

6.2.7. Determination of the Effect of MZ on Phase II Carcinogen Metabolising Enzymes

6.2.7.1. Effect of MZ on Glutathione-S-Transferase (GST) Activity

Twenty four rats were randomly divided into four groups (6 animals/group). Animals in group I was kept as untreated (normal). Group II animals received sunflower oil only (vehicle control). Animals in group III and IV received MZ 50 and 250 mg/kg body wt respectively. MZ was administered orally once daily for 15 days. On the 16th day one hour after carotenoid administration animals were sacrificed and liver was excised. Liver homogenate (25%) was prepared in ice cold Tris buffer (PH 7.4, 0.1M). GST activity was estimated by the method of Habig et al (Chapter 2, section 2.2.8.7).

6.2.7.2. Effect of MZ on UDP-Glucoronyl Transferase Activity

UDP-glucoronyl transferase activity was estimated by the method of Issalbacher (Issalbacher et al 1962) modified by Hollman (Hollman and Touster 1962).

Incubation mixture containing 0.5 ml of Tris HCl buffer (1M, pH 7.4), 0.2 ml of Triton X-100, 0.05ml of MgCl2 (50 mM), 0.05 ml of p-nitrophenol, 0.18 ml of water, and 0.1 ml of liver homogenate (1 mg of protein) from MZ pre-treated and untreated animals of the above experiment was incubated at 37°C for 2 minutes. After incubation, the reaction was started by the addition of 0.1 ml of UDP-glucuronic acid (30 mM). The reaction was arrested at different time points (0, 10 and 15 minute) by
adding 2 ml of ice cold trichloroacetic acid (5%, w/v) into tubes containing 100 µl of reaction mixture. After brief centrifugation, 1 ml of supernatant solution was made alkaline by adding 0.25 ml of 2M NaOH and the absorbance was read at 450 nm using spectrophotometer. The activity of UDP-glucoronyl transferase was expressed as nano moles/min/mg protein.

6.3. RESULTS
6.3.1. Effect of MZ on Induction of Hepato Cellular Carcinoma by NDEA

The morphological analysis of the livers of control animals showed a number of tumour nodules on the surface with variable shapes, normal morphology was completely lost and necrotic mass was seen at some places. The livers of 50 and 250 mg/kg b.wt MZ treated animals showed fewer incidences of tumour nodules and they retained the normal morphology of the liver (Figure 6.1).

The effect of administration of MZ on the survival rate of hepatocellular carcinoma harbouring animals is shown in Table 6.1. There was a significant increase in the survival of animals by MZ treatment. On 29th week after NDEA administration none of the animals in both 50 and 250 mg/kg b.wt MZ treated groups died due to carcinoma. But in NDEA alone treated control group 3 out of 6 animals died due to carcinoma. On 29th week all the animals in control groups developed HCC but in 50 mg/kg b.wt MZ treated group 3 out of 6 animals and in 250 mg/kg b.wt MZ treated group only 1 out of 6 animals developed HCC. Administration of NDEA significantly elevated the liver weight of all the control group animals. Oral administration of MZ was found to be very effective in reducing the increased liver weight in a dose dependent manner (Table 6.1).

6.3.2. Effect of MZ on Serum and Liver Parameters during NDEA Induced Hepato Cellular Carcinoma

The activity of major marker enzymes of hepatic function viz ALP, GPT and GOT were drastically elevated in both serum and liver tissue of NDEA alone treated control animals and these elevated levels were significantly decreased by MZ treatment in a dose dependent manner. The activity of the enzyme γ-glutamyl transpeptidase (γ-GT), a marker of cellular proliferation was found to be significantly elevated in the serum and the liver of NDEA alone treated and vehicle control
Figure 6.1. Effect of MZ on the morphology of liver during NDEA induced hepatocellular carcinoma

Normal

NDEA control

NDEA+50 mg/kg b.wt MZ

NDEA+250 mg/kg b.wt MZ
Table 6.1. Effect of MZ on Survival Rate, Tumour Incidence and Liver Weight of NDEA Induced HCC Harboring Animals (on 29\textsuperscript{th} week)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals Survived (on 29\textsuperscript{th} week)</th>
<th>Tumor incidence (on 29\textsuperscript{th} week)</th>
<th>Liver weight (g)/100g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6/6</td>
<td>0/6</td>
<td>3.1±0.5</td>
</tr>
<tr>
<td>NDEA Control</td>
<td>3/6</td>
<td>6/6</td>
<td>6.2±1.1***</td>
</tr>
<tr>
<td>NDEA+ Sunflower oil (Vehicle control)</td>
<td>4/6</td>
<td>6/6</td>
<td>6.1±0.92\textsuperscript{ns}</td>
</tr>
<tr>
<td>NDEA+ MZ (50 mg/kg b.wt)</td>
<td>6/6</td>
<td>3/6</td>
<td>4.15±0.78**</td>
</tr>
<tr>
<td>NDEA+ MZ (250 mg/kg b.wt)</td>
<td>6/6</td>
<td>1/6</td>
<td>3.8±0.622***</td>
</tr>
</tbody>
</table>

The values were expressed as mean ± standard deviation (SD); n=6.

The mean values were statistically analyzed by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnett’s multiple comparison test) using Graph pad Instat 3 Software.

\textsuperscript{ns} - Not significant (p>0.05). \textsuperscript{*} p <0.01 and \textsuperscript{**} p <0.001 significant. Significant level of NDEA alone treated control group was compared with that of normal group, whereas significant levels of MZ-treated and vehicle control groups were determined by comparing with those of NDEA alone treated control group.
animals. Administration of MZ at different doses significantly lowered the elevated levels of \( \gamma \)-GT in a dose dependent manner. The level of total bilirubin, which is increased in both the serum and the liver tissue by NDEA, was also decreased by the treatment of MZ (Table 6.2 and 6.3).

6.3.3. Histopathological Analysis of the Liver of NDEA Treated Rats

Histopathology of liver of NDEA alone treated control group animals showed carcinomatous growth composed of groups of large pleomorphic polyhedral or oval cells having hyperchromatic nuclei. There were many degenerating cells and mitotic cells. Stroma showed areas of necrosis and inflammatory reaction. Many congested blood vessels and areas of hemorrhage were seen. All these carcinomatous changes were found to be significantly decreased by MZ pretreatment in a dose dependent manner (Figure 6.2). In the highest dose MZ treated groups hepatocytes retained normal morphology. The portal areas, kuffer cells and sinusoidal spaces were also appeared normal indicating the protective potential of MZ against NDEA induced HCC in rats.

6.4. Effect of MZ Administration on 3-MC Induced Sarcoma

6.4.1. Tumour Incidence

The effect of MZ on tumour incidence i.e. on development of 3-MC-induced sarcoma is shown in figure 6.3 and figure 6.4. In 3-MC alone treated control group, animals started developing sarcoma on the 6\(^{th}\) week after carcinogen administration and within 18 weeks all the mice were found to develop sarcoma. Animals treated with the lowest dose MZ (50mg/kg b.wt) started developing sarcoma only on 15\(^{th}\) week after 3-MC treatment. In 250mg/kg b.wt MZ treated group, only one animal developed sarcoma on 18\(^{th}\) week indicating lower incidence of sarcoma development at higher dose of MZ. On 30\(^{th}\) week, 12 out of 15 in the 50mg/kg b.wt MZ treated animals and only 6 out of 15 animals in the 250mg/kg b.wt MZ groups were found to develop sarcoma. The size of the tumour in the MZ treated animals were found to be very small when compared to that of the 3-MC control animals.

6.4.2. Survival of Animals

The effect of MZ on survival of animals treated with 3-MC is given in figure 6.5. Animals in the 3-MC control group started dying due to tumour burden from the
Table 6.2. Effect of MZ on Serum Parameters during NDEA Induced Hepatic Carcinoma

<table>
<thead>
<tr>
<th></th>
<th>SGOT (IU/l)</th>
<th>SGPT (IU/l)</th>
<th>ALP (KA)</th>
<th>Bilirubin (mg/dl)</th>
<th>γ-GT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>31±4</td>
<td>35±4</td>
<td>28±2</td>
<td>0.6±.7</td>
<td>29±3</td>
</tr>
<tr>
<td>NDEA Control</td>
<td>427±31***</td>
<td>290±18***</td>
<td>66±1***</td>
<td>2.7±.2***</td>
<td>106±10***</td>
</tr>
<tr>
<td>NDEA+Sunflower oil (Vehicle control)</td>
<td>420±30ns</td>
<td>292±15ns</td>
<td>60±1.8ns</td>
<td>2.4±0.24ns</td>
<td>100±8ns</td>
</tr>
<tr>
<td>NDEA+MZ (50mg/kg b.wt)</td>
<td>226±14***</td>
<td>130±10***</td>
<td>49±2***</td>
<td>1.6±.1***</td>
<td>68±4***</td>
</tr>
<tr>
<td>NDEA+MZ (250mg/kg b.wt)</td>
<td>92±10***</td>
<td>78±12***</td>
<td>34±3***</td>
<td>1.1±.1***</td>
<td>43±6***</td>
</tr>
</tbody>
</table>

The values were expressed as mean ± standard deviation (SD); n=6. The mean values were statistically analyzed by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnett’s multiple comparison test) using Graph pad Instat 3 Software.

ns - Not significant (p>0.05), *** p<0.001 significant. Significant level of NDEA alone treated control group was compared with that of normal group, whereas significant levels of MZ-treated and vehicle control groups were determined by comparing with those of NDEA alone treated control group.
Table 6.3. Effect of MZ on Liver Parameters during NDEA Induced Hepatic Carcinoma

<table>
<thead>
<tr>
<th></th>
<th>GOT (IU/mg protein)</th>
<th>GPT (IU/mg protein)</th>
<th>ALP (KA/mg protein)</th>
<th>γ-GT (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>41.3±4.3</td>
<td>32.6±1.5</td>
<td>15.3±4.7</td>
<td>0.695±.1</td>
</tr>
<tr>
<td>NDEA Control</td>
<td>320.7±16***</td>
<td>171±33***</td>
<td>58±4***</td>
<td>5±2***</td>
</tr>
<tr>
<td>NDEA+Sunflower oil (Vehicle control)</td>
<td>306±4 ns</td>
<td>165±28 ns</td>
<td>50±3.2 ns</td>
<td>4.2±1 ns</td>
</tr>
<tr>
<td>NDEA+MZ (50mg/kg.b.wt)</td>
<td>131±25***</td>
<td>67±8***</td>
<td>28±3***</td>
<td>1.3±2***</td>
</tr>
<tr>
<td>NDEA+MZ (250mg/kg.b.wt)</td>
<td>45±5***</td>
<td>45±3***</td>
<td>19±2***</td>
<td>0.66±0.4***</td>
</tr>
</tbody>
</table>

The values were expressed as mean ± standard deviation (SD); n=6. The mean values were statistically analyzed by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnett’s multiple comparison test) using Graph pad Instat 3 Software.

ns - Not significant (p>0.05), ***p<0.001 significant. Significant level of NDEA alone treated control group was compared with that of normal group, whereas significant levels of MZ-treated and vehicle control groups were determined by comparing with those of NDEA alone treated control group.
Figure 6.2. Histopathological analysis of NDEA treated liver

Normal

NDEA control

Vehicle control

NDEA+ 50mg MZ

NDEA+250mg MZ

(Hemotoxylin – Eosin staining, x 400).
Figure 6.3. Effect of MZ on 3-MC induced sarcoma

3-MC alone treated

3-MC+50 mg/kg b.wt MZ  3-MC+250 mg/kg b.wt MZ
Figure 6.4. Effect of MZ on tumor incidence of mice treated with 3-MC

Figure 6.5. Effect of MZ on the survival rate of 3-MC induced sarcoma in mice
8th week after 3-MC treatment and all the animals were died by 20th week. All the animals in the 50 and 250 mg/kg b.wt MZ treated groups were alive even after 16 and 20 weeks respectively after 3-MC treatment. In the 50 mg/kg b.wt MZ treated group, 5 out of 15 animals and in the 250 mg/kg b.wt MZ treated group, 10 out of 15 animals were found to be alive when the experiment was completed. These results indicated that MZ significantly decreased tumour development and increased survival of animals.

6.5. Effect of MZ Administration on DMBA and Croton oil Induced Two-stage Skin Carcinogenesis in Mice

Effect of MZ administration on two-stage carcinogenesis was studied by two methods. In the first method MZ was administered before DMBA application and in the second method MZ was administered before promotion by croton oil.

6.5.1. Effect of MZ on Tumour Initiation

Topical application of MZ before DMBA was found to enhance the tumour latency period. In the control animals the first tumour appeared on the 6th week after DMBA application where as in mice treated with MZ at doses 0.1, 0.2 and 1%, the first tumour appeared only on 8th, 10th and 12th weeks respectively. MZ administration also reduced the tumour incidence (Figure 6.6). In the control group, all the eight animals developed tumours on the 20th week. But in MZ treated groups (0.1, 0.2 and 1%/mouse), the number of mice with papillomas were reduced to 5, 3 and 3 respectively [Figure 6.7(a)]. The number of papillomas per mouse was also found to be decreased by MZ administration. The average number of tumours per mouse on the 20th week was 7.5±3.3 in the control group but MZ treatment at doses 0.1, 0.2 and 1% /mouse reduced the number to 4.4±1.8, 4±1.5 and 3.33±1.4 respectively [Figure 6.7(b)].

6.5.2. Effect of MZ on Tumour Promotion

Topical application of MZ prior to croton oil administration resulted in significant protection against skin tumour promotion in a dose dependent manner. The administration of MZ at doses 0.1, 0.2 and 1%/mouse increased the tumour latency period from 6 weeks of control group animals to 10 weeks, 12 weeks and 12 weeks respectively. There was also a dose dependent reduction in tumour incidence.
Figure 6.6. Effect of MZ on DMBA and croton oil induced two-stage skin carcinogenesis in mice

DMBA + Croton oil

DMBA + Croton oil + 1 % MZ (Prior to DMBA)  
DMBA + Croton oil + 1 % MZ (Prior to croton oil)
Figure 6.7. Effect of MZ on tumor initiation during DMBA and croton oil induced two-stage skin papillomagenesis

(a) Number of animals developed papilloma

(b) Number of papilloma developed per mice
All the 8 animals in the carcinogen treated control group developed papillomas on 20th week. But in mice treated with MZ at doses 0.1, 0.2 and 1%, only 5, 4 and 3 animals developed papillomas respectively by 20th week [Figure 6.8 (a)]. The average number of papillomas per tumour bearing mice, which was 7.5±3.3 numbers in control group animals on 20th week, was reduced by the administration of MZ at doses 0.1, 0.2 and 1%/mouse to 5.2±2.2, 4.5±1.8 and 2.67±1.4 numbers respectively [Figure 6.8 (b)].

Application of DMBA or croton oil alone did not produce any tumours suggesting that 470 nano mole DMBA/mouse was ineffective to induce carcinogenesis without promotion by croton oil. Furthermore animals topically treated with DMBA, croton oil and sunflower oil (group IV) were found to produce tumours like the control group animals indicating that sunflower oil itself could not inhibit papilloma formation.

6.6. Inhibition of Different Isoforms of Microsomal Cytochrome P450 Enzymes by MZ In vitro

Effect of MZ on various CYP450 enzymes in vitro was studied by using microsomal preparation from phenobarbitone (a CYP450 inducer) treated animals. It was found that all the CYP450 enzymes were significantly inhibited by MZ in a concentration dependent manner. Concentration needed for 50 % inhibition (IC_{50}) of CYP1A2 (MROD) was 5 µg/ml, CYP2B 1/2 (PROD) was 8 µg/ml and CYP1A1 (EROD) was 12 µg/ml while that of CYP 2E1 (aniline hydroxylase) was 7µg/ml and CYP 1A, 2A, 2B, 2D and 3A (aminopyrene-N-demethylase) was 10.5 µg/ml (Figure 6.9).

6.7. Inhibition of Different Isoforms of Microsomal Cytochrome P450 Enzymes by MZ In vivo

In normal animals, the activities of PROD, MROD and EROD were 4.4±2.1, 6.1±3 and 7.1±1 nano moles of resorufin formed/min/mg protein respectively. In phenobarbitone alone treated control animals, the activities of these three CYP450 isoenzymes were increased to 24±5.1, 18.4±6 and 16±3.2 nano moles of resorufin formed/min/mg protein respectively. The increase in the activities of CYP1A1 (PROD) was 6 fold, CYP1A2 (MROD) was 3 fold and CYP2B1/2 (EROD) was 2
Figure 6.8 Effect of MZ on tumor promotion during DMBA and croton oil induced two-stage skin papillomagenesis

(a) Number of animals developed papilloma

(b) Number of papilloma developed per mice
fold. Oral administration of MZ significantly inhibited phenobarbitone induced CYP450 isoenzymes in a dose dependent manner. In 50 mg/kg body wt MZ treated group, the activities of PROD, MROD and EROD were 7±3.2, 6.9±2.8 and 10±1.4 nano moles of resorufin formed/min/mg protein respectively; and in 250 mg/kg body wt MZ treated group the activities of all these isoenzymes were reduced to 3.9±2, 5.4±2.2 and 6.8±0.98 nano moles of resorufin formed/min/mg protein respectively which were almost close to those of normal animals (Figure 6.10).

6.8. Effect of MZ Administration on Phase II Enzymes

UDP-glucuronyl transferase (UDPGT) activity of normal animals was 26.79±0.9 nano moles/min/mg protein and this was increased by MZ pre-treatment (50 and 250 mg/kg b.wt) to 34.93±0.7 and 55.6±1.5 nanomoles/min/mg protein respectively. GST activity was also increased to 75.1±2.3 and 130.8±3.1 nano moles/min/mg protein by MZ pre-treatment (50 and 250 mg/kg b.wt respectively) when compared to that of normal animals which was 37.6±1.1 nanomoles/min/mg protein (Figure 6.11).

6.9. DISCUSSION

In the present study, anti-carcinogenicity of MZ was studied against 3-MC induced sarcoma model. 3-MC was found to be metabolized in liver by microsomal CYP450 enzymes to several oxygenated metabolites mainly dihydrodiols and epoxides. These active electrophillic carcinogenic species interact with critical cellular target molecules and thereby cause genetic damage (Wood et al., 1978.). Results of this study revealed that MZ at doses 50 and 250 mg/kg b.wt markedly inhibited 3-MC-induced sarcoma development in mice. The reliable criterion for judging the value of any anticancer drug is the prolongation of life span of the animal and reduction of tumour incidence. The present results demonstrated reduction in sarcoma development and increase in life span of tumour bearing mice treated with MZ in a dose dependent manner. The reduced life span of 3-MC control mice was evidently due to excessive tumour burden. It can therefore be inferred that MZ increased the life span of sarcoma bearing mice by preventing tumour progression, possibly by preventing genetic damage. The reduced tumour growth and increased
Figure 6.9 Effect of MZ on Phase 1 carcinogen metabolizing enzymes \textit{in vitro}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.9.png}
\caption{
IC_{50} values:
- MROD 5 \(\mu\)g/ml,
- PROD 8 \(\mu\)g/ml,
- EROD 12 \(\mu\)g/ml,
- Aniline hydroxylase 7 \(\mu\)g/ml,
- Aminopyrene-N-demethylase 10.5 \(\mu\)g/ml.
}
\end{figure}

Figure 6.10. Effect of MZ on CYP450 enzyme activity \textit{in vivo}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.10.png}
\caption{
PB- Phenobarbitone, VC- vehicle control.
Values are expressed as mean \(\pm\)SD; \(n=6\). Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. Values are significant at *** \(P<0.001\) against PB control groups.
}
\end{figure}
Figure 6.11. Effect of MZ administration on phase II enzymes

UDP GT - UDP-glucuronyl transferase
GST- Glutathione-S-Transferase

Values are expressed as mean ±SD; n=6. Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. Values are significant at * P <0.05, *** P< 0.001 against normal group.
survival of tumour bearing mice suggested the delaying impact of MZ on carcinogenesis.

NDEA is an indirect acting carcinogen and requires metabolic activation by CYP450 enzymes to form ethyl radical (ultimate carcinogen). Ethyl radical so formed attack the DNA and produce genetic changes which in turn results in carcinogenesis (Boitier, 1995). In this study we checked anticarcinogenic effect of MZ against NDEA induced hepatocellular carcinoma. In the NDEA alone treated animals there was a drastic elevation in the levels of hepatic marker enzymes like AST, γ-GT, ALT and ALP in both serum and liver tissue and were significantly reduced to normal levels by MZ treatment. Treatment of MZ also reduced the tumour incidence when compared to the control group. Histopathological analysis further supported the anticarcinogenic potential of MZ.

Anti-carcinogenicity of MZ was also studied on two-stage skin carcinogenesis model induced by 7, 12 dimethyl benz(a)anthracene (DMBA) as initiator and croton oil as promoter. DMBA undergoes extensive metabolism in liver and the parent compound itself is not thought to be toxic. The well characterized metabolic pathway begins with oxidation by CYP450 (primarily CYP1A1 and 1B1) to arene oxides, which can be hydrolyzed by microsomal epoxide hydrolase to dihydrodiols, which can be further oxidized to highly reactive diol epoxides by CYP1A1 and CYP1B1 (Kleiner et al., 2004). The diol epoxides, which can form DNA adducts, are thought to be the ultimate carcinogens (Shimada et al., 2004). The classical skin tumour promoter-12-O-tetradecanoylphorbol-13-acetate (TPA) is the active component of croton oil. In keratinocytes, TPA increases the production of ROS and hydroperoxides which promote skin tumourigenesis (Joyce and Fischer, 2010). In this study it was found that skin papilloma produced by DMBA and croton oil application was significantly inhibited by topical application of MZ. We can see that treatment of MZ before DMBA application inhibited the papilloma formation indicating that MZ could inhibit the initiation of carcinogenesis by DMBA. Treatment of MZ before croton oil application was also found to inhibit the papilloma formation indicating that MZ has profound anti-tumour promotional activity.
Chemoprevention is an effective means of cancer control. Chemo-preventive agents are either blocking agents or suppressing agents. These agents can be further sub-divided into bifunctional inducers (inducing both phase I and phase II enzymes), monofunctional inducers (induce phase II enzymes only) and dual-acting agents (inhibit phase I enzymes and induces phase II enzymes) (Henderson et al., 2000). In order to understand the possible anti-carcinogenic activity of MZ, its effect on phase I carcinogen metabolizing enzymes were studied. The results indicated that MZ could significantly inhibit different CYP450 isoenzymes which are involved in the activation of chemical carcinogens. This could be one of the mechanisms of action of MZ against chemical carcinogenesis.

Another major mechanism of protection against carcinogenesis is mediated by the induction of enzymes involved in their detoxification. Phase II enzymes such as glutathione-S-transferases (GSTs), uridine diphosphate glucuronyl transferases (UDPGT) etc are the major enzymes involved in the detoxification process. Transcriptional control of the expression of phase II enzymes is mediated through the antioxidant response element (ARE) found in the regulatory regions of their genes. The binding of transcription factor Nrf2 to ARE in response to treatment with certain phyto chemicals appears to be essential for the induction of prototypical phase II enzymes (Talalay and Fahey, 2001). Present study revealed that MZ pre-treatment significantly elevated the activities of different phase II enzymes like GST and UDPGT in a dose dependent manner. This ability of MZ possibly based on inducing the activation of Nrf2 and the expression of antioxidant enzymes.

Our previous studies showed that MZ could effectively scavenge singlet oxygen (Firdous and Kuttan, 2012), superoxide radical, hydroxyl radical and nitric oxide radical at very low concentrations (Firdous et al., 2010a). The stable free radicals like diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS) were also found to be scavenged by MZ. Oral administration of MZ for one month was found to increase levels of antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase as well as GSH levels in mice (Firdous et al., 2010b). Hence the anti-carcinogenic activity of the carotenoid MZ can also be comes from the scavenging of oxygen radicals produced
during carcinogenesis. Our studies also reported the anti-mutagenic activity of MZ against both direct acting mutagens and mutagens needing microsomal activation using *Salmonella typhimurium* strains (Ames test) (Firdous *et al.*, 2010b). These results substantiate the present data on the inhibition of chemical carcinogenesis by MZ.

In conclusion, we can say that MZ is a “dual-acting agent” as this carotenoid inhibited specific CYP450 isoenzymes and at the same time it augmented the detoxification process through induction of phase II detoxification enzymes. Prevention of promotional events in the mouse skin can be attributed to the free radical scavenging mechanism of this carotenoid. By the virtue of its protective activities like antioxidant activity, singlet oxygen quenching effect, anti-inflammatory activity, anti-mutagenesity, antitumour effects, induction of apoptosis in transformed cells, inhibition of specific CYP450 isoenzymes, induction of phase II enzymes as well as anti-carcinogenesity, MZ can be considered as an ideal candidate for the class of chemopreventive agents.