Chapter-V

Evaluation of neoplastic potential of mancozeb and its underlying mechanism in human keratinocyte HaCaT cells and mouse skin: [6]-gingerol as an antidote
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5.1. Introduction

Mancozeb has several animal studies revealing its carcinogenic risk (Shukla et al., 1990; Monis and Valentich, 1993; Belpoggi et al., 2002). But its potential for human carcinogenicity has been so far inadequately evaluated. Thus, owing to the extensive and often indiscriminate usage of this fungicide and the prospective hazards involved, therein, it is an imperative need to determine its neoplastic potential and the underlying molecular mechanism in human cells so that the preventive measures against it can be devised. As skin is a major portal of entry and a vulnerable target of pesticides, we selected this organ for our study. In the earlier chapter (Chapter IV) we had shown that mancozeb has genotoxic potential in human skin keratinocyte HaCaT cells as well as in in vivo Swiss albino mouse skin (Chapter IV). As a reasonable correlation between the mutagenic potential of numerous chemical agents and their carcinogenicity has been reported in many studies, findings of mancozeb’s mutagenic effects in HaCaT cells inspired us to test for its possible neoplastic potency as well. In this chapter the neoplastic potential of mancozeb in human skin keratinocyte (HaCaT) cells and its underlying molecular mechanism involving oxidative stress generation, NF-κB, extracellular signal regulated kinase ½ (ERK1/2) and Plk1 signaling pathways is illustrated. Then, we validated the involvement of these molecules in underlying mechanism of mancozeb-induced hyperproliferation in in vivo system of Swiss albino mouse skin also. Its skin carcinogenic potential has already been proved by us earlier in Swiss albino mouse (Shukla et al., 1990).

Furthermore, the potential of [6]-gingerol to inhibit mancozeb-induced neoplastic alterations is also demonstrated in this chapter. [6]-Gingerol, a well reported chemopreventive antioxidant, has shown commendable protective capacity against mancozeb-induced oxidative stress and genotoxicity in the earlier chapter (Chapter IV).

First three objectives of this thesis have been dealt with in this chapter.

5.1.1. Inflammation-mediated cancer

In case of cancer, inflammation is now a well recognized risk factor (Vagefi and Longo, 2005; Aggarwal et al., 2006; Peek and Crabtree, 2006). Chronic inflammation has been associated with many steps of tumorigenic process, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Coussens and
Several pro-inflammatory gene products such as TNF, interleukins (IL)-1a, IL-1b, IL-6, chemokines, MMP-9, VEGF and COX-2 have been discovered to be critically involved in inhibition of apoptosis, proliferation, angiogenesis, invasion, metastasis and other carcinogenic processes. Many of these genes are chiefly regulated by the transcription factor NF-κB, which is constitutively active in majority of cancers.

5.1.1.a. NF-κB

NF-κB is an ubiquitous factor implicated in the control of many vital processes as varied as immune (Baeuerle and Henkel, 1994) and inflammatory responses (Barnes and Karin, 1997), apoptosis (Bours et al., 2000) and cell proliferation (Karin et al., 2002). These last two attributes expound the involvement of NF-κB in the tumorigenic course as well as the promise of a targeted therapeutic intervention (Baeuerle and Baltimore, 1996).

NF-κB, present in an inactive state in most of the cells, gets activated in cancer cells by various inflammatory stimuli and carcinogens, and the inflammatory gene products controlled by this transcription factor mediate tumorigenesis (Aggarwal, 2004; Shishodia and Aggarwal, 2004). Loercher’s group demonstrated the function of NF-κB in the gene expression modulations with cell transformation, development and malignancy of the murine Squamous Cell Carcinoma (SCC) (Loercher et al., 2004).

5.1.1.b. MAPK

MAPK family of proteins performs key signal transduction functions in eukaryotic cells. MAPKs are necessary for the cellular responses to virtually all kinds of extracellular stimuli (Cobb et al., 1994; Kyriakis and Avruch, 2001). In mammalian cells, the three best characterized MAP kinase pathways are the ERK, stress-activated protein kinase (SAPK)-1/ JNK and SAPK-2/p38 α and β (p38) pathways (Kyriakis and Avruch, 2001). Whereas the ERK pathway is preferentially activated by mitogens (Cobb et al., 1994), both the JNK and p38 MAPK cascades are generally responsive to stress and inflammatory cytokines (Kyriakis and Avruch, 2001) which has got them both the more suitable name of stress-activated protein kinases (SAPKs) (Kyriakis and Avruch, 2001). In turn, once activated, MAPKs trigger numerous cellular responses, which range from proliferation to apoptosis and inflammation. Interestingly, it is observed that the relative expressions and activities of different isoforms within each MAPK subfamily can alter the cellular response towards different outcomes. Among ERKs, ERK1 and 2 are known to be activated by mitogenic stimuli but not much is known about the stimulus selectivity of the atypical ERK members ERK3 to ERK8 (Coulombe and Meloche, 2006). The role of
p38α in cancer is quite well known while nothing much is known about the role of other p38 isoforms p38β, p38γ and p38δ.

There are some reports which show that pesticides can modulate the phosphorylation of these MAPKs. Diethyldithiophosphate (DEDTP) is a metabolite formed by biotransformation of organophosphorous (OP) pesticides. DEDTP has been found to induce phosphorylation of ERK, JNK and p38, and, NFAT nuclear translocation in human CD4+ T lymphocytes (Esquivel-Sentíes et al., 2010).

5.1.1.c. COX-2

COX-2, the inducible isoform of prostaglandin-H-synthase with expression controlled by NF-κB and ERK (Liu et al., 2003), has shown involvement in the development of a variety of human cancers including malignant epithelial cancers (Goulet et al., 2003). Expression of COX-2 in human cancers can be stimulated by diverse growth factors, cytokines, oncogenes, and other factors (Aggarwal et al., 2006). Enhanced COX-2 expression has been recorded in 91% of SCCs (Zimmermann et al., 1999) and 93% of melanomas (Denkert et al., 2001).

5.1.1.d. E twenty-six (ETS)-like transcription factor 1 (Elk1)

Elk1, a member of the ETS oncogene family, is a key nuclear mediator of Ras-ERK MAPK pathway-induced proliferation and cell survival (Vickers et al., 2004). The transcriptional activity of Elk-1 is stimulated via phosphorylation by MAPKs and main function of Elk-1 is to regulate the expression of growth-related proteins in response to extracellular stimuli (Whitmarsh et al., 1997; Babu et al., 2000; Boros et al., 2009). Expression studies have demonstrated that elevated expression of Elk1-like protein is involved in development of human esophageal SCC (Chen et al., 2006a). Pesticide DDT and its metabolites are also reported to enhance the transcriptional activity of Elk1 (Bratton et al., 2009).

5.1.1.e. Cyclin D1

Cyclin D1 is a G1-specific cyclin that is reckoned to be the mitogenic sensors that transmit signals from the extracellular environment to the central cell cycle machinery (Sherr and Roberts, 1999). Among all G1 regulatory molecules, the cyclin D1/CDK4 complex is observed to be deregulated very frequently in various cancers and is contemplated to contribute directly to neoplastic transformation and growth (Kim and Diehl, 2009). Combined treatment of 17beta estradiol (estrogen) with organophosphorous
pesticides malathion and parathion induced Cyclin D1- mediated cell transformation of human breast epithelial cells \textit{in vitro} (Calaf and Roy, 2008).

5.1.1.f. S100 proteins

S100 protein family is a multigenic group of non-ubiquitous, cytoplasmic EF-hand Ca$^{2+}$-binding proteins (Donato, 1999). Many genes of this family are present in the epidermal differentiation complex (EDC) located on chromosome 1q21 (Volz et al., 1993; Hardas et al., 1996; Wicki et al., 1996) and thus many S100 proteins are found to be expressed in the human epidermis or in cultured keratinocytes (Broome et al., 2003; Wolf et al., 2003). Hermani et al. (2006) indicated their involvement in inflammatory and neoplastic disorders as most S100 genes are found near a break-point region on human chromosome 1q21, which if affected, is a frequent target for chromosomal rearrangements which occur during tumor development. One protein of this family S100B has already found a role in the clinical setting for diagnosis and therapeutic monitoring of malignant melanoma (Salama et al., 2008).

5.1.1.f.1 S100A9

S100A9 also known as calgranulin B, is characterized by cell type-specific expression in cells of epithelial, myeloid and endothelial origin and accretion in conditions of acute and chronic inflammatory conditions (reviewed by Donato, 2001). S100A9 may have a significant function in promoting and/or responding to the hyperproliferative state in the epidermis, as its expression is strongly elevated within the first week subsequent to epidermal injury (Thorey et al., 2001). Furthermore, exposure to tumor promoter in mouse skin enhances S100A9 level and glucocorticoid treatment suppresses this increase (Christoffer et al., 2002). These studies suggest that S100A9 may play a role in epidermal response to tissue injury, inflammation and carcinogenesis.

5.1.1.f.2 S100A6

S100A6 also known as calcyclin, is a protein whose expression gets augmented on growth factor treatment in human fibroblasts (Calbretta et al., 1985) and its knockdown by using antisense S100A6 has been shown to inhibit proliferation in fibroblasts (Breen and Tang, 2003). S100A6 is involved in many diverse processes such as cell cycle, intracellular calcium homeostasis and signaling, ion transport, exocytosis of insulin from pancreatic cells, cytoskeletal rearrangement, and ubiquitinated proteolytic degradation (Filipek et al., 2002; Breen and Tang, 2003; Nowotny et al., 2003). S100A6 is found to be elevated in many cancers including malignant melanoma (Maelandsmo et al., 1997).
5.2.1. Chemicals and reagents

Mancozeb, propidium iodide (PI), Bay11-7082, PD98059, 5-Bromo-2'-deoxyuridine (BrdU), DCHF-DA dye, DMSO, 3-methylcholanthrene (3-MCA), bovine serum albumin (BSA), phenylmethyl sulfonyl fluoride, [6]-gingerol and NAC were from Sigma-Aldrich (St. Louis, Columbia, USA). Cyclin D1, Plk1, COX-2, phospho-NF-κB p65 (Ser 536) (p-NF-κB p65), phospho-Elk1(ser 383) (p-Elk1) and phospho-ERK1/2 (p44/42) (p-ERK1/2) antibodies were procured from Cell Signaling Technology, Inc. (Danvers, MA, USA) while antibodies for BrdU, proliferating cell nuclear antigen (PCNA) S100A6 and S100A9 were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC) and horseradish peroxidase conjugated anti-mouse/anti-rabbit secondary antibodies were procured from Bangalore Genei (Bangalore, India) and Cell Signaling Technology, Inc. (Danvers, MA, USA), respectively. Ribonuclease (RNase) was from Bangalore Genei (Bangalore, India) while polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA). Rest of the chemicals used in the study were of analytical grade of purity and procured locally.

5.2.2 Experiment I: In vitro system

5.2.2.1. Cell culture and treatments

HaCaT cells were procured from NCCS, Pune, India and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1% penicillin streptomycin (Gibco Lifetech, Karlsruhe, Germany) in a humidified atmosphere of 95% O2 and 5% CO2 at 37°C. NAC was dissolved in distilled water while mancozeb, nocodazole, Bay11-7082 (NF-κB inhibitor), PD98059 (ERK1/2 inhibitor) and 3-MCA were dissolved in DMSO and then all the compounds were diluted in medium to attain the desired final concentrations. DMSO was used as the vehicle control. The final volume of DMSO added to cells did not exceed 0.5% (v/v). [6]-Gingerol was dissolved in minimal amount of ethanol and then suitably diluted in the medium to achieve desired final concentration for treatment of cells. 3-MCA was used as a positive control in cell transformation assays. Treatments with [6]-gingerol, NAC, Bay11-7082, PD98059 and Plk1 siRNA (siPlk1) were given 1h prior to the addition of mancozeb and maintained during the whole incubation period. 20 μM and 5 μM doses of PD98059 and Bay11-7082 were selected on the basis of reports by Cui et al. (2004) and Ren et al. (2006), respectively. For selection of dose of 3-MCA, dose-range of 0.5-5 μg/ml was tested in proliferation assay and amongst these doses 2.5 μg/ml was found to induce maximum proliferation. TPA was used as a positive control in expression studies for p-ERK 1/2 and its downstream proteins as Zhou et al. (2005) have
previously shown that 100 ng/ml TPA gives significant activation of ERK1/2. On the basis of earlier published results (Kee et al., 2002) nocodazole (33 µM for 1 h) was used as positive control in Western blotting of Plk1. TNF-α (20ng/ml for 10 mins), a known inducer of NF-κB (Cho et al., 2007), was used as positive control in Western blotting for p-NF-κBp65.

5.2.2.2. RNA interference

Transfection of cells with Plk1 (GenBank accession no. NM_005030) siRNA sense sequence 5'-GGAGGUUCGCGGCAAGtt-3'; antisense sequence 5'-CUUGCCCGAACCUCUtt-3' (Ambion, Austin, TX) (siPlk1), targeting regions of the Plk1 transcript at exon 1, was performed by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of Tyagi et al. (2010). Briefly, 3 X 10⁴ cells were seeded on 24-well plates in normal medium without antibiotics and transfected at 30 to 40% confluence. Transfection complex was prepared, with desired concentration from 20 µM stock siRNA and 2.5 µl of Lipofectamine 2000 in 100 µl Opti-MEM I medium (Invitrogen, Carlsbad, CA) per well. 100 µl of newly formed transfection complexes were incubated for 25 min at room temperature and then added to wells. Next, 150 µl Opti-MEM1 was poured in the wells, making the total volume to 250 µl Opti-MEM1 medium per well. 150 µl of DMEM F12 medium with 20% FBS was added to wells after 18 h. After testing the knockdown efficiency of various siPlk1 concentrations 30, 50, 75 and 100 nM at different time durations 12 h, 24 h, 36 and 48h through reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting, siPlk1 concentration 100 nM and 48h time duration were selected for the study. 24h post-transfection cells were exposed to mancozeb. A scrambled siRNA was purchased from Ambion (Negative control siRNA #1) (Ambion, Austin, TX) and used as the control.

5.2.2.3. Cell proliferation assay

To select noncytotoxic mancozeb dose for cell proliferation assay, effect of various doses of mancozeb on cell viability was assessed. 1 × 10⁴ cells suspended in 100 µL of complete culture medium were plated in each well of 96-well microtiter plates and exposed to mancozeb concentrations (0.05-5 µg/ml). After incubation for 24, 48 and 72 h at 37°C, the effect of mancozeb on cell viability was assessed by Neutral Red Uptake assay (Borenfreund and Puerner, 1985) taking vehicle control cells as 100% viable.

For quantitative determination of cellular proliferation, 1x10³ HaCaT cells suspended in 100 µl DMEM were plated in each well of 96-well microtiter plates and cultivated for 24h,
prior to addition of mancozeb. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. After exposing to mancozeb doses 0.25, 0.5, 1, 1.5, 2.0 and 2.5 µg/ml for 3 days, medium was replaced with fresh medium and cells were further incubated for 5 more days. After total 8 days, MTT (5 mg/ml) 20 µl/well was then added for 5 h. After careful removal of the medium, 0.1 ml of buffered DMSO was added to each well, and plates were shaken. The absorbance of mancozeb-exposed and vehicle control cells were recorded on a microplate reader (Biotek Instruments Inc., Winooski, VT, USA) at the wavelength of 530 nm.

5.2.2.4. BrdU incorporation assay

DNA-synthesis, which correlates well with cellular proliferation, was measured by flow cytometric BrdU incorporation analysis according to the slightly modified protocol of Ramos et al. (2005). In brief, cells were exposed to mancozeb for 24 h with 10 µM BrdU being added to the cells for the final 18 h. Cells were then harvested and fixed overnight in 80% ethanol at −20 ºC. Briefly, fixed cells were incubated with 0.5 mg/ml RNase A for 10 min at 37 ºC, followed by 0.25% paraformaldehyde for 30 min at room temperature i.e., 25ºC. Cells were incubated with 0.1M HCl and 0.5% triton X-100 for 10 min at 4ºC. DNA was denatured by boiling samples for 10 min, and blocking for 1 h with 1% BSA. After overnight incubation with anti-BrdU antibody, secondary IgG-FITC antibody was added for 45 min. Samples were acquired and analyzed on flow cytometer [Becton-Dickinson LSR (BDLSR) II, San Jose, CA, USA] using ‘CellQuest’ software.

5.2.2.5. Cell cycle analysis

For cell cycle analysis, the cells were prepared as described earlier (Arora et al., 2006). Briefly, control and mancozeb-exposed cells (for 24h) were washed with PBS and centrifuged at 200 g for 10 min at 4ºC. The pellet was fixed in 1 mL of 70% ice-cold ethanol for 30 min. and resuspended in 50 µg/mL PI with RNase A (100 µg/mL) followed by incubation for 30 min in dark. The samples were acquired and analyzed on flow cytometer BDLSR II (San Jose, CA, USA) using ‘CellQuest’ software.

5.2.2.6. Immunofluorescence studies

Immunofluorescence for ERK1/2, JNK, p38α, BrdU and PCNA was carried out. HaCaT cells were incubated with mancozeb for 30 mins. in case of ERK1/2, JNK and p38α while for BrdU and PCNA the incubation period with mancozeb was 24h. For immunofluorescence study of ERK1/2, JNK and p38α, 30 mins. duration was selected because among all tested durations of 30 mins, 60 mins, 120 mins. and 24h, ERK1/2
activation was seen only at 30 mins. whereas there was no change with respect to control at the other durations. This exposure was followed by fixing with 4% paraformaldehyde in PBS for 7 min, permeabilization with 0.1% Triton-X-100 in PBS for 7 min, and blocking with 3% BSA in PBS for 1 h with gentle agitation. After blocking, cells were washed in PBS and incubated with pERK1/2, JNK, p38α, BrdU and PCNA antibodies for 1 h at room temperature, followed by the incubation with and secondary IgG-FITC antibody for 1 h at room temperature. After washing, fluorescence microscopy was performed using Olympus IX51 (Olympus America Inc., Center Valley, PA, USA) and images acquired with the help of software Image Pro Express.

5.2.2.7. Anchorage-independent growth assay

Ability of mancozeb to induce HaCaT cells to grow as anchorage independent colonies in soft agar was determined by using modified protocol of Ouyang et al. (2008). Briefly, 1 x 10⁶ cells were exposed to mancozeb in 1 ml of 0.33% Basal Medium Eagle (BME) (Invitrogen, Carlsbad, CA) agar containing 15% FBS over 3.5 ml of 0.5% BME agar containing 15% FBS in each well of 6-well plate. After 3 days exposure, fresh medium was added every alternate day. The cultures were maintained in 37°C, 5% CO₂ for 14 days, and then the cell colonies were scored using Olympus IX51 microscope (Olympus America Inc., Center Valley, PA, USA).

5.2.2.8. In vitro cell transformation assay

The standard in vitro cell transformation assay was performed according to the method suggested by IARC/NCI/EPA Working Group (1985) using the procedure developed by Ahn et al. (2008) with slight modifications. Actively growing cells were seeded at a density of 10⁴ cells/25 cm² flask in 5ml of culture medium. 24 hr after plating, culture medium was removed from each flask and replaced with medium containing test compounds (mancozeb and 3-MCA). After 3 days exposure the medium was replaced with fresh medium and changed twice a week during the following 4 weeks. Most of the cells were detached after 2-3 days of confluency. Thus, HaCaT cells were re-seeded at a density of 10⁴ cells/ 25 cm² flask at 2 weeks after test compound treatment. At the end of incubation the medium was removed and the cells were rinsed with saline, fixed in methanol and stained with 5% aqueous Giemsa for scoring growing attached colonies.

5.2.2.9. Total cell Lysate, nuclear and cytosolic cell lysates preparation

After completion of treatment, 3–5×10⁶ cells were washed with 0.5 ml PBS and allowed to stand on ice in chilled buffer (50mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 20mM NaF,
100mM Na$_3$VO$_4$, 0.5% NP-40, 1% Triton X-100, 1mM PMSF, pH 7.4) with freshly added protease inhibitor cocktail (Protease inhibitor Cocktail Set III; Calbiochem, La Jolla, CA, USA) for a few seconds followed by scraping on ice. The lysed cells were incubated in ice for 30 minutes and collected as total cell lysate. Extracts of nuclear and cytosol were prepared according to the modified method of Afaq et al., (2003). Briefly, after treatments, 3–5×10$^6$ cells were washed with 0.5 ml PBS and suspended in 20 µl of buffer A; containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl$_2$, 0.5 mM DTT, and 0.1% NP-40, per 1×10$^6$ cells; allowed swell on ice for 10 min; mixed; and then centrifuged at 12,000×g at 4°C for 10 min. Supernatant was taken as cytosolic fraction. The nuclear pellet obtained was resuspended in 15 µl of buffer B; containing 20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 5 µg/ml leupeptin; incubated on ice for 15 min; and centrifuged at 12,000 × g for 10 min at 4°C. The supernatant, extract, was transferred to new tubes. These were either used immediately or stored at -80 °C for later use.

5.2.2.10. Protein estimation

The protein estimation was performed by the Folin-Phenol method as mentioned in section 4.2.2.d. of this dissertation.

5.2.2.11. Western blotting

To assess the protein expression Western blotting was performed as described by (Towbin et al., 1979). Cytosolic fraction was used to analyse S100A6, S100A9, Plk1, p-Elk1, COX-2, Cyclin D1 and β-actin while nuclear fraction was used for the analysis of p-NF-κB p65. 50-100 µg protein was aliquoted in a 1.5 ml eppendorf tube and equal amount of 2x loading dye (62 mM 4x Stacking gel buffer, 5% β-Mercaptoethanol, 3% SDS, 20% Glycerol, 0.1% Bromophenol blue and sterile distilled water) was added, the samples were mixed and boiled at 100° C for 4 minutes and centrifuged for 5 seconds to collect the total protein in the bottom of the tube. The proteins were resolved over 10-18 % Tris-Glycine gels (depending on the molecular weight of the protein to be analysed) by SDS poly acrylamide gels electrophoresis (SDS-PAGE) (Bangalore Genei, India) at 110-120 volts for about 90 min (or until the gel has run off unneeded (Tris-HCl, Glycine and SDS) and transferred onto a PVDF membrane, sandwiching the gel and membrane soaked in 1x transfer buffer (containing Tris-HCl, Glycine and Methanol) (Sponge – 3mm thick filter paper - gel - membrane - filter paper – sponge) using Semi-dry electro-blot transfer assembly at a constant voltage (15 volts) for 20-40 minutes. The non-specific sites were blocked by incubating the blot with 5% non-fat dry milk in buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 1 h at room temperature or overnight at 4°C. The
blot was washed with wash buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) thrice for 10 minutes each and then incubated for 1 h with appropriate primary antibody specific for the protein to be assessed. The antibodies were used at dilutions specified by the manufacturer. The blot was washed for 2x10 minutes, 2x5 minutes and then incubated with the corresponding secondary antibody HRP-conjugate (Bangalore Genei, India) at 1:1000 dilutions for 2 h at 4°C. The blot was washed for 2x10 minutes, 3x5 minutes. The complex formed by the protein and the antibody was detected using chemiluminescence kit from Millipore and visualized by Versa Doc Imaging System (BioRad Model 4000, Hercules, CA). Densitometric measurements of the bands were done with digitalized scientific software UN-SCAN-IT automated digital system version 5.1 purchased from Silk Scientific Corporation (Orem, USA). The intensity of the bands then given in terms of relative pixel density for each band normalized to the band of β-actin.

5.2.3. Experiment II: *In vivo* system

5.2.3.1. Animal treatment

Swiss albino mice (female, 20-22 g body weight) were obtained from Indian Institute of Toxicology Research animal breeding colony and acclimatized for 1 week. The ethical approval for the experiment was obtained from institutional ethical committee. They were kept under standard laboratory conditions (temperature 23±2°C, relative humidity 55±5%), and, were fed with synthetic pellet basal diet (Ashirwad, Chandigarh, India) and tap water *ad libitum*. Protective efficacy of [6]-gingerol against mancozeb-induced damage was compared with that provided by NAC. Table 5.1. illustrates the treatment schedule of different groups. The animals were divided into 12 groups consisting of 4 animals each. Same doses as in section 4.2.3.1. of this dissertation have been used and the rationale for dose selection has been mentioned therein.

Table 5.1. Treatment Schedule to study hyperproliferation induced by mancozeb in mouse skin and protection afforded by [6]-gingerol pre-treatment

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TOPICAL TREATMENT</th>
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<tbody>
<tr>
<td>Gr. I</td>
<td>Vehicle control</td>
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<tr>
<td>Gr. II</td>
<td>[6]-Gingerol (I) (20µM/animal) alone</td>
</tr>
<tr>
<td>Gr. III</td>
<td>[6]-Gingerol (II) (30µM/animal) alone</td>
</tr>
<tr>
<td>Gr. IV</td>
<td>[6]-Gingerol (III) (40µM/animal) alone</td>
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<tr>
<td>Gr.</td>
<td>Treatment Details</td>
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<tr>
<td>V</td>
<td>Mancozeb (Single topical application, 100 mg/kg b.wt)</td>
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<tr>
<td>VI</td>
<td>[6]-Gingerol (I) (20µM/animal) + Mancozeb (100 mg/kg b.wt)</td>
</tr>
<tr>
<td>VII</td>
<td>[6]-Gingerol (II) (30µM/animal) + Mancozeb (100 mg/kg b.wt)</td>
</tr>
<tr>
<td>VIII</td>
<td>[6]-Gingerol (III) (40µM/animal) + Mancozeb (100 mg/kg b.wt)</td>
</tr>
<tr>
<td>IX</td>
<td>NAC (I) (30µM/animal) alone</td>
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<tr>
<td>X</td>
<td>NAC (II) (40µM/animal) alone</td>
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<tr>
<td>XI</td>
<td>NAC (I) (30µM/animal) + Mancozeb (100 mg/kg b.wt)</td>
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<tr>
<td>XII</td>
<td>NAC (II) (40µM/animal) + Mancozeb (100 mg/kg b.wt)</td>
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5.2.3.2. Tissue preparation

After 24h of exposure, the animals were sacrificed humanely and skin from the treatment site of the interscapular region was taken off with the help of scissors and transferred into pre-cooled dishes. Hair was removed with sharp scalpel blades, and subcutaneous fat was scraped off on ice. For flow cytometric analysis of BrdU incorporation, cell cycle distribution, protein expression and ROS generation, single cell suspensions of skin tissue were prepared using Medimachine (Beckton Dickinson, San Jose, USA) as described earlier (Shukla and Arora, 2001) while for Western blotting skin tissues were minced and homogenized. For hyperplasia study, the skin was removed and immediately fixed in freshly prepared 10% buffered formalin at 4°C.

5.2.3.3. BrdU incorporation assay

Twenty-four h before sacrificing the animals, they were injected intraperitoneally with BrdU (125mg/kg in 0.9% NaCl) according to the protocol by Sharma et al. (2009). Cell suspensions were prepared for flow cytometric analysis as mentioned earlier in section 5.2.2.4. of this dissertation. Samples were then acquired and analyzed on flow cytometer BDLSR II (San Jose, CA, USA) using ‘CellQuest’ software.

5.2.3.4. Cell cycle analysis

For the flow cytometric cell cycle analysis, cell suspensions from skin tissues were prepared as described earlier in section 5.2.2.5. of this dissertation. The samples were then acquired and analyzed on flow cytometer BDLSR II (San Jose, CA, USA) using ‘CellQuest’ software.
5.2.3.5. Hyperplasia

For the epidermal hyperplasia study, 6h after the last treatment, skin from the painted area was excised carefully and fixed immediately in 10% buffered formalin for 72 hrs. The formalin fixed tissue samples were deformalized in running tap water for 7-8h and then dehydrated in ascending grades of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100% each for 2h) using automated tissue processor (STP120 Microm, Germany), cleared in xylene and embedded in paraffin. Vertical tissue sections (5 micron) were cut using microtome (Leica RM 2155, Germany), stained with haematoxylin and eosin. The stained slides were examined and imaged under Olympus IX51 microscope (Olympus America Inc., Center Valley, P.A., U.S.A.) equipped with Image Pro Express software as collective system. Epidermal hyperplasia was determined as mean vertical epidermal thickness and mean number of vertical epidermal cell layers by microscopic examination of different treated skin tissue sections. For each section of skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at 10 equal distance interfollicular sites using light microscope equipped with an eyepiece micrometer. 10 sites per skin section per sample were examined per group (a total of four skin samples were determined in a group). The number of nucleated cell layers was counted in the same areas.

5.2.3.6. Preparation of nuclear and cytosolic cell lysates

Cytosolic and nuclear extracts were prepared according to the modified protocol of Serpi et al., (1999). After the excision of the skin tissue samples they were homogenized for 15 min in 1.5 ml of cytoplasmic lysis buffer (containing 20% glycerol, 20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, 100 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A and 1 µg/ml antipain) on ice, then centrifuged for 4 min at 2000 r.p.m. To the pellet, 1 ml of nuclear lysis buffer was added (500 mM instead of 10 mM NaCl) and incubated for 30 min in an ice bath. The samples were then centrifuged for 15 min at 15,000 r.p.m. at 4°C and the supernatant (nuclear extract) was collected.

5.2.3.7. Protein estimation

The protein estimation was performed by the Folin-Phenol method as mentioned in section 4.2.2.d. of this dissertation.

5.2.3.8. Western blotting

After quantification of proteins by the Lowry’s method Western blotting was carried out in the cytosolic and nuclear fractions of skin tissue as described previously in section 5.2.2.11. Cytosolic fraction was used to analyse S100A6, S100A9, p-Eik1, COX-2, Cyclin
D1 and β-actin; nuclear fraction was used for the analysis of p-NF-κB p65. The intensity of the bands was measured using software UNSCAN-IT automated digital system version 5.1 (Orem, USA) and then given in terms of relative pixel density for each band normalized to the band of β-actin.

5.2.3.9. Protein expression analysis by flow cytometry

Flow cytometric analysis of ERK1/2 protein expression was carried out as mentioned earlier (Bhui et al., 2010). Following treatments, the cells were collected by scraping and centrifugation. They were resuspended briefly in 0.5-1 ml PBS and formaldehyde was added to a final concentration of 2% for 10 minutes at 37°C for fixation. The tubes were chilled on ice for 1 minute. To remove fix prior to permeabilization, cells were pelleted by centrifugation and resuspended in 90% methanol followed by incubation of 30 minutes on ice. 2-3 ml of incubation buffer (0.5 g BSA in 100mL 1X PBS) was added to each tube and rinsed by centrifugation. The cells were resuspended cells in 100 μl incubation buffer per assay tube and blocked for 10 mins. at room temperature. Then the primary antibody (phospho and total ERK1/2) was added at the appropriate dilution suggested by the manufacturer to the assay tubes and incubated for 30-60 minutes at room temperature. The cells were once again rinsed as before in incubation buffer by centrifugation. This was followed by resuspension of cells in FITC-conjugated secondary antibody, diluted in incubation buffer according to the manufacturer’s recommendations and incubation for 30 minutes at room temperature. Finally, the cells were resuspended in 0.5 ml PBS and analyzed on flow cytometer.

5.2.4. Statistical analysis

All data are expressed as mean ± SD of three independent experiments. For the statistical analysis of all data, Student’s t-test was used and p<0.05 was considered as significant.

5.3. Results

5.3.1. Experiment I: In vitro system

5.3.1.1. Mancozeb- induced proliferation and DNA synthesis in HaCaT cells

As mancozeb at doses higher than 2μg/ml reduced cell viability of HaCaT cells, MTT proliferation assay was carried out at doses 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml of mancozeb. These six increasing doses of mancozeb increased cell proliferation by 1.25±0.04, 2.08±0.12, 1.84±0.05, 1.68± 0.07, 1.52±0.1 and 1.32±0.04 fold as compared to control cells (Figure 5.1.A). Since 0.5μg/ml dose exposure was found to induce maximum increase in cell proliferation, thus this proliferative dose was used in all assays henceforth.
Figure 5.1. A. MTT cell proliferation assay in Mz\textsuperscript{a}-exposed cells. B. Representative histograms of cell cycle assay of (a) control, (b) Mz\textsuperscript{a} (0.5µg/ml)-exposed cells. X-axis corresponds to fluorescence of PI while Y-axis is event counts. Bar diagram in the right panel showing the S-fraction of cells obtained in cell cycle assay. C Representative histograms of flow cytometric analysis of BrdU incorporation in (a) control, (b) Mz\textsuperscript{a} (0.5µg/ml)-exposed cells. X-axis corresponds to fluorescence of BrdU while Y-axis is event counts. The percentage of cells falling within the phases G0/G1–G2/M (left region, M1) and S (right region, M2) are indicated. Bar diagram in the right panel showing percentage of BrdU incorporation. Immunofluorescence photomicrographs of D. BrdU expression and E. PCNA expression in control and Mz\textsuperscript{a} (0.5µg/ml)-exposed HaCaT cells. Green fluorescence indicates BrdU or PCNA levels in cells. Data shown is the results of 3 independent experiments with similar results. The data were significant at p<0.05. *shows significant increase over control.

\textsuperscript{a}Mz- mancozeb
In order to examine the affect on distribution of cells among various cell cycle phases, flow cytometric cell cycle analysis was carried out. Significant increase in S-phase fraction of cells to 34.51±2.28% in case of mancozeb exposed cells as compared to vehicle control (7.76±0.51%) was seen (p<0.05) (Figure 5.1.B.). In flow cytometric BrdU incorporation assay 7.53±1.17% cells were found to be BrdU positive in vehicle control which got increased to 34.67±1.60% on mancozeb exposure (Figure 5.1. C.). Thus, flow cytometric analysis of both, BrdU incorporation as well as cell cycle profiles showed significant ~5-fold increase in the proportion of cells in S-phase in mancozeb exposed cultures (p<0.05). Furthermore, cells were analyzed by fluorescence microscopy for BrdU incorporation (Figure 5.1.D.) and PCNA (Figure 5.1.E.) expression. Increased PCNA expression (Figure. 5.1.E.) and high proportion of BrdU stained cells (Figure 5.1D.) indicated an increase in DNA synthesis rate in mancozeb-exposed cells over control cultures.

5.3.1.2. Mancozeb- induced cell transformation of HaCaT cells

In colony forming assay, spontaneous formation of colonies in vehicle control averaged 2.32±0.82 colonies per flask while 3-MCA (positive control) induced 22.5±3.62 colonies/flask. Mancozeb exposure induced 16.47±2.43 colonies/ flask thereby giving significant ~7 fold increase over control (p<0.05) (Figure 5.2.A.). In anchorage independent growth assay, compared with 22.3±4.04 colonies in the vehicle control, mancozeb and 3-MCA exposures significantly (p<0.05) increased the colony number to 142.7±15.01 and 237±28, respectively (Figure 5.2.B.). These results indicate that mancozeb-exposed HaCaT cells obtained ~6-fold increase in the ability of anchorage-independent growth for colony formation in soft agar.

5.3.1.3. Mechanism underlying mancozeb-induced transformation in HaCaT cells involves oxidative stress and inflammatory proliferation related proteins p-NF-κBp65, p-ERK1/2 and Plk1

Mancozeb’s potential to induce oxidative stress has already been shown earlier (Chapter IV). To validate whether this oxidative stress underlies behind mancozeb-induced proliferation, NAC, a known antioxidant, was given to cells prior to mancozeb exposure. NAC could inhibit mancozeb-induced proliferation as seen by ~52% and ~56% reduction in BrdU incorporation (Figure 5.3.) and S-phase fraction in cell cycle (Figure 5.4 A.) over those in mancozeb-alone exposed cells, respectively. Likewise, mancozeb-induced cell transformation was also repressed by ~50% on NAC treatment (Figure 5.4 B.), thus indicating the role of mancozeb-generated oxidative stress in the proliferation and transformation.
Figure 5.2. A. Bar diagram showing results of colony forming assay in control, Mz\textsuperscript{a} and 3-MCA\textsuperscript{b} exposed cells. Lower panel shows representative photomicrographs. B. Bar diagram showing results of anchorage-independent growth assay in control, Mz\textsuperscript{a} and 3-MCA\textsuperscript{b} exposed cells. Lower panel shows representative photomicrographs. Data shown is the result of 3 independent experiments with similar results. The data were significant at p<0.05. *shows significant increase over control.\textsuperscript{a} Mz: mancozeb, \textsuperscript{b}3-MCA: 3-Methyl cholangrene.
Figure 5.3. A. Bar diagram showing the percentage of BrdU incorporation in HaCaT cells. B. Representative histograms of flow cytometric analysis of BrdU incorporation in (a) control, (b) Mzb, (c) [6]-Ga+Mzb both and (d) NAC+Mzb both treated HaCaT cells. X-axis corresponds to fluorescence of BrdU while Y-axis is event counts. The percentage of cells falling within the S phase (right region, M1) are indicated. Data shown is the result of 3 independent experiments with similar results. The data were significant at p<0.05. * and ** show significant changes over control and Mzb-exposed groups, respectively. (Histograms of groups [6]-Ga-alone and NAC-alone are not shown).

Figure 5.4. A. Bar diagram showing the S-fraction of cells obtained in cell cycle assay of HaCaT cells. Lower panel shows representative histograms of cell cycle assay of (a) control, (b) Mz\textsuperscript{a}, (c) siPlk1+Mz\textsuperscript{a} both, (d) PD98059/ Mz\textsuperscript{a} both, (e) Bay11-7082+ Mz\textsuperscript{a} both, (f) NAC+ Mz\textsuperscript{a} both treated cells. X-axis corresponds to fluorescence of PI while Y-axis is event counts. * and ** show significant change over control and Mz\textsuperscript{a}-exposed group, respectively. (Figures of groups NAC, PD98059, siPlk1 and Bay11-7082 are not shown here).

\textsuperscript{a} Mz- mancozeb
Mancozeb exposure was found to modulate various inflammation and proliferation related proteins. Both mancozeb and TPA induced ERK1/2 activation, as seen by increase in nuclear translocated p-ERK1/2 (Figure 5.5. A.) while no change with respect to control was seen in JNK and p38α immunofluorescence (Figure 5.5. B & C.). In immunoblot analysis for p-NF-κBp65 expression, noteworthy up-regulation by ~1.82 and ~2.34 fold over vehicle control was seen in mancozeb-(24h exposure) and TNF-α-exposed HaCaT cells, respectively (Figure 5.5. D.). Plk1 which is an important cell cycle regulatory protein increased by ~2.2 fold on mancozeb exposure. Immunoblotting for proliferation associated ERK1/2’s downstream Elk1, cyclin D1 and COX-2 was also carried out (Figure 5.5.D.). Considerable increases of ~1.6 and ~1.9 fold in phosphorylated form of Elk1 was noted in mancozeb (30 min exposure) and TPA exposed cells (Figure 5.5.D.). Concomitant up-regulation of ~1.98 and ~3.12 fold in Cyclin D1 protein expression while increase of ~2.57 and ~3.59 fold was seen in COX-2 protein expression on mancozeb (24h exposure) and TPA exposure in HaCaT cells, respectively (p<0.05) (Figure 5.5.D.). In a simultaneous study conducted in our lab, comparative analysis of protein expression profiles of mancozeb (200 mg/ kg b.wt) exposed and vehicle treated mouse skin revealed that S100A6 and S100A9, known markers of keratinocyte differentiation and proliferation-regulated proteins, were significantly upregulated on mancozeb exposure (p<0.05)(Tyagi et al., 2011). Thus in this study we have evaluated the involvement of S100A6 and S100A9 proteins in mancozeb-induced hyperproliferation in HaCaT cells. NF-κB is known to play important regulatory role in expression of S100A6 and S100A9. Expression levels of S100A6 and S100A9 were also found to be increased on mancozeb-exposure in HaCaT cells. Significant increase of ~1.86 and ~ 2.41 fold in S100A6 and ~1.77 and ~2.47 fold in the expression of S100A9 proteins was recorded in mancozeb (24h exposure) and TPA exposed HaCaT cells, respectively (p<0.05) (Figure 5.5. D.).

To validate the involvement of p-NF-κBp65, p-ERK1/2 and Plk1, these proteins were specifically inhibited followed by studying the affects of these inhibitions on mancozeb-induced proliferation. Treatment with PD98059, siPlk1 and Bay11-7082 individually could inhibit mancozeb-induced proliferation as seen by significant reduction in the S-phase fraction by ~52%, ~49% and ~53%, respectively (p<0.05) (Figure 5.4.A.). Similarly, mancozeb-induced cell transformation also was reduced significantly by ~42%, ~35% and ~41% on inhibition of ERK1/2, Plk1 and NF-κBp65 proteins, respectively (p<0.05) (Figure 5.4.B.). These results validated the role of these inflammation and proliferation related proteins in mancozeb-induced proliferative response.
Figure 5.5. A. Immunofluorescence photomicrographs showing subcellular localization of ERK1/2 in control, Mz\textsuperscript{a}- and TPA-exposed HaCaT cells. Immunofluorescence photomicrographs showing subcellular localization of B. JNK1/2 and C. p38 α in control and Mz\textsuperscript{a}-exposed HaCaT cells. Green fluorescence indicates ERK1/2, JNK1/2 and p38 α expression in cells. D. Western blot analysis of p-NF-κBp65, p-Elk1, Cyclin D1, COX-2, Plk1, S100 A6 and S100A9 in control and Mz\textsuperscript{a}-exposed HaCaT cells. Lane 1: vehicle control, lane 2: Mz\textsuperscript{a}-exposed cells. For p-NF-κBp65 and Plk1 blots, lane 3 are TNF-α-exposed and nocodazole-exposed HaCaT cells while in p-Elk1, Cyclin D1, COX-2, S100A6 and S100A9 blots lane 3 is TPA-exposed HaCaT cells, respectively. Right panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. Data shown is the result of 3 independent experiments with similar results. The data were significant at p<0.05. \*shows significant increase over control.

\textsuperscript{a}Mz:Mancozeb
5.3.1.4. [6]-Gingerol inhibited mancozeb-induced HaCaT cell transformation

Pre-treatment with [6]-gingerol at doses 10, 20 and 30µM 1 h prior to mancozeb exposure, notably reduced mancozeb-induced cell proliferation by ~15%, ~29% and ~40%, respectively, in a dose-dependent manner (p<0.05). Out of the three doses of [6]-gingerol 30 µM dose gave the maximum reduction of ~40% in cell proliferation over mancozeb-alone exposed cells (p<0.05) (Figure 5.6.A) and thus, this dose had been selected for further assays. This protection afforded by [6]-gingerol was further verified by PCNA expression and BrdU incorporation assays where marked decrease in PCNA expression (Figure 5.6.B.) and ~66% reduction in BrdU stained cells (Figure 5.3.), respectively, on [6]-gingerol administration as compared to that in mancozeb-alone exposed cells was observed (p<0.05).

Mancozeb-induced increase in S-phase fraction in cell cycle assay also got restrained by ~73% on [6]-gingerol pre-treatment (Figure 5.6.C.). Similarly, mancozeb-induced anchorage independent growth potential was also inhibited by [6]-gingerol pre-treatment as seen by ~68% reduction in no. of colonies in anchorage independent growth assay over mancozeb-alone exposed cells (Figure 5.6.D.), respectively.

Mancozeb-induced up-regulation of proteins p-NF-κBp65, Plk1, p-Elk1, COX-2, Cyclin D1, S100A6 and S100A9 was restrained when [6]-gingerol pre-treatment was given (Figure 5.7.A). Expression levels of proteins p-NF-κBp65, Plk1, p-Elk1, COX-2, Cyclin D1, S100A6 and S100A9 were reduced by ~1.4, ~1.6, ~1.34, ~1.69, ~1.43, ~1.51 and ~1.4 fold in [6]-gingerol pre-treated cells in comparison to mancozeb-exposed cells (Figure 5.7.A.). Mancozeb-induced nuclear translocation of p-ERK1/2 (activation) was also markedly inhibited by [6]-gingerol treatment (Figure 5.7.B.).

5.3.2. Experiment II : In vivo system

5.3.2.1. Mancozeb-induced hyperproliferation in mouse skin

Mancozeb-induced cell proliferation in mouse skin was evident by ~5 fold enhancement in the percentage of BrdU incorporation into newly synthesized DNA to 36.89±3.76 as compared to the control group (6.87±0.51) (Figure 5.8.A.). Similar increase over control was observed in the S-phase fraction of cell cycle in mancozeb-exposed group (Figure 5.8.B.).

Skin histology of mancozeb-exposed mice showed induction of mild epidermal hyperplasia with epidermal thickness increasing to 55.6 ± 7.9 µm over 19.2 ± 2.1 µm thickness in control group (Figure 5.9., Table 5.2.).
Figure 5.6. A. MTT cell proliferation assay in control, Mz\(^a\) and [6]-G\(^b\)+Mz\(^a\) both treated HaCaT cells. B. Immunofluorescence photomicrographs of PCNA expression in (a) control, (b) Mz\(^a\), (c) [6]-G\(^b\) (30μM)+Mz\(^a\) both and (d) [6]-G\(^b\) (30μM) treated HaCaT cells. Green fluorescence indicates PCNA levels in cells. C. Bar diagram showing the percentage of cells in S-phase fraction of cell cycle in HaCaT cells. Right panel shows representative histograms of flow cytometric cell cycle analysis of BrdU incorporation in (a) control, (b) Mz\(^a\), (c) [6]-G\(^b\) (30μM)+Mz\(^a\) both treated HaCaT cells. X-axis corresponds to fluorescence of PI while Y-axis is event counts. D. Bar diagram showing results of anchorage-independent growth assay HaCaT cells. Right panel shows representative photomicrographs of (a) control, (b) Mz\(^a\), (c) [6]-G\(^b\) (30μM)+Mz\(^a\) both treated cells. Data shown is the result of 3 independent experiments with similar results. The data were significant at \(p<0.05\). * and ** show significant changes over control and Mz\(^a\) exposed cells, respectively. (Figures of [6]-G\(^b\)- alone group are not shown in C & D).

\(^a\)Mz:-Mancozeb, \(^b\)[6]G-[6] Gingerol
Figure 5.7. A Western blot analysis of p-NF-κBp65, p-Elk1, Cyclin D1, COX-2, Plk1, S100 A6 and S100A9 in HaCaT cells. Lane 1: vehicle control, lane 2: mancozeb-exposed cells, lane 3: [6]-gingerol+ mancozeb and lane 4: [6]-gingerol-treated cells. Right panel shows fold change calculated with respect to control on the basis of pixel density measured by UNSCAN-IT software. B. Immunofluorescence photomicrographs showing subcellular localization of ERK1/2 in HaCaT cells. Green fluorescence indicates ERK1/2 expression in cells. Data shown is the result of 3 independent experiments with similar results; representative figures shown. The values were significant at p<0.05. * and # show significant changes over control and mancozeb-exposed groups, respectively.
Topical application of mancozeb to mouse skin resulted in a significant development of oedema with $23.5 \pm 1.71 \text{mg}$ weight of skin punch as compared to $14.2 \pm 0.92 \text{mg}$ in control group ($p<0.05$) (Table 5.2).

Table 5.2. Skin oedema and histological alterations induced by mancozeb in mouse skin and protection afforded by [6]-gingerol pre-treatment

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Weight of skin punch$^a$ (mg)</th>
<th>Epidermal thickness$^b$ (µm)</th>
<th>Number of epidermal layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>$14.2 \pm 0.92$</td>
<td>$19.2 \pm 2.1$</td>
<td>1–3</td>
</tr>
<tr>
<td>[6]-Gingerol III (40 µM/animal)</td>
<td>$13.9\pm 1.5$</td>
<td>$18.5 \pm 2.6$</td>
<td>1–3</td>
</tr>
<tr>
<td>NAC II (40 µM/animal)</td>
<td>$14.5 \pm 1.5$</td>
<td>$19.5 \pm 2.6$</td>
<td>1–3</td>
</tr>
<tr>
<td>Mancozeb (100mg/kg b wt.)</td>
<td>$23.5 \pm 1.71^*$</td>
<td>$55.6 \pm 7.9^*$</td>
<td>3–6</td>
</tr>
<tr>
<td>[6]-Gingerol III (40 µM/animal) + Mancozeb (100mg/kg b wt.)</td>
<td>$16.3 \pm 1.3^{**}$</td>
<td>$25.9 \pm 2.9^{**}$</td>
<td>1–4</td>
</tr>
<tr>
<td>NAC II (40 µM/animal)+ Mancozeb (100mg/kg b wt.)</td>
<td>$18.3 \pm 1.5^{**}$</td>
<td>$33.5 \pm 3.2^{**}$</td>
<td>1–5</td>
</tr>
</tbody>
</table>

$^a$ Data represents the mean $\pm$ SD of 4 mice in each group. $^b$ Each value represents the mean $\pm$ SD of 10 regions in each slide. The values were significant at $p<0.05$.

* shows significant increase over control. ** shows significant decrease over mancozeb group.
Figure 5.8. A. Bar diagram showing BrdU incorporation in mouse skin DNA. Lower panel shows representative histograms of flow cytometric analysis of BrdU incorporation in (a) control, (b) Mzb, (c) [6]-Ga (III)+ Mzb both and (d) NAC+Mzb both treated mouse skin DNA. X-axis corresponds to BrdU-fluorescence while Y-axis is event counts. The percentage of cells in phases G0/G1–G2/M (left region, M1) and S (right region, M2) are indicated. Values shown are the result of 3 independent BrdU incorporation assays with similar results. B. Bar diagram showing the S-fraction of cells obtained in cell cycle assay of mouse skin. Lower panel shows representative histograms of cell cycle assay of (a) control, (b) Mz⁵, (c) [6]-Ga(III) +Mzb both and (d) NAC(II)+Mzb both treated mouse skin. X-axis corresponds to PI-fluorescence while Y-axis is event counts. Values shown are the result of 3 independent cell cycle assays with similar results. Data were significant at p<0.05. * and ** show significant change over control and Mzb-exposed groups, respectively. (Histograms of groups [6]-Ga(I)+Mzb, [6]-Ga(II)+ Mzb, NAC(I) +Mzb, [6]-Ga(III)- alone and NAC(II)-alone are not shown).

Figure 5.9. Epidermal hyperplasia in skin sections processed for H&E staining (magnification ×10). Sections of skin treated with DMSO (vehicle control) (a), section of skin exposed to mancozeb showing mild hyperplasia with increase in epidermal thickness (b), section of skin pretreated with [6]-gingerol (40µM/animal) showing less epidermal thickness (c) section of skin pretreated with NAC (40µM/animal) showing less epidermal thickness (d), section of skin treated with [6]-gingerol (40µM/animal) only (e) and section of skin treated with NAC (40µM/animal) only (f).
5.3.2.2. Mechanism underlying mancozeb-induced hyperproliferation in mouse skin involves oxidative stress and inflammatory proliferation related proteins p-NF-κBp65, p-ERK1/2 and Plk1

Involvement of oxidative stress generation in mancozeb-induced neoplastic alterations in HaCaT cells has been demonstrated in section 5.3.1.3. of this dissertation. To validate whether this oxidative stress underlies behind mancozeb-induced hyperproliferation in mouse skin as well, NAC topical pre-treatment in mouse skin was given 1 h prior to mancozeb exposure. NAC thwarted mancozeb-mediated enhancement of BrdU incorporation and S-phase fraction of cell cycle in a dose-dependent manner (Figure 5.8.A. & 5.8.B.). The percentage of DNA BrdU incorporation decreased from 36.89±3.76% in mancozeb-exposed animals to 25.51±2.71% and 17.68±1.42% in NAC pre-treated animals at the two increasing doses 30 and 40µM/ animal of NAC, respectively (Figure 5.8.A.). Similarly, cell fraction in S-phase of cell cycle was also dose-dependently reduced from 35.93±2.57% in mancozeb-exposed group to 25.19±2.71% and 19.08±1.24% in NAC-pre-treated groups at two increasing doses of NAC, respectively (Figure 5.8.B.). NAC also showed significant protection against skin hyperplasia and oedema induced by mancozeb (p<0.05) (Figure 5.9., Table 5.2.). Epidermal thickness and weight of skin punch reduced from 55.6 ± 7.9µM and 23.5 ±1.71 mg in mancozeb-alone exposed group to 33.5 ± 3.2 µM and 18.3 ± 1.5 mg in 40 µM NAC pre-treated group, respectively (Figure 5.9., Table 5.2.). Thus, this efficacy of NAC in protecting against mancozeb-induced proliferation indicated that oxidative stress generated in mancozeb-exposed mouse skin is the underlying mechanism for hyperproliferation.

Mancozeb exposure was found to modulate various inflammation and proliferation related proteins. On mancozeb exposure fluorescence representing p-ERK1/2 expression increased by ~3 folds over control (Figure 5.10.A.). In immunoblotting, up-regulation of ~3.59, ~2.25, ~2.87, ~4.07, ~2.53, ~2.55 and ~2.95 fold in the expression of p-NF-κBp65, Plk1, p-Elk1, COX-2, Cyclin D1, S100A6 and S100 A9 proteins on mancozeb exposure was recorded (Figure 5.10.B).

5.3.2.2. [6]-Gingerol inhibited mancozeb-induced hyperproliferation in mouse skin

Pre-treatment with [6]-gingerol at doses of 20, 30 and 40µM/ animal prior to mancozeb exposure notably reduced mancozeb-induced hyperproliferation in a dose-dependent manner. Significant (p<0.05) decrease in the percentage of DNA BrdU incorporation to 22.9±1.81%, 19.55±1.36% and 11.01±1.13% as compared to mancozeb-alone group
(36.89±3.76%) was noted on three increasing doses of [6]-gingerol, respectively (Figure 5.8.A).

Similarly, mancozeb-mediated increase in S phase fraction of cell cycle also got thwarted significantly from 35.93±2.56% to 25.24±1.95%, 20.42±1.74% and 11.95±1.12% by three
increasing doses of [6]-gingerol (p<0.05) (Figure 5.8.B). Epidermal hyperplasia and skin oedema induced by mancozeb were also markedly inhibited by [6]-gingerol application prior to mancozeb exposure (Figure 5.9.). [6]-Gingerol (40µM/ animal) application significantly abridged epidermal thickness and weight of skin punch to 25.9 ± 2.9µM and 16.3 ± 1.3mg as compared to 55.6 ± 7.9 µM and 23.5 ± 1.71 mg in mancozeb-alone exposed animals (p<0.05). Furthermore, this protection potency afforded by [6]-gingerol was better than that by NAC.

Mancozeb-induced upregulation in proteins p-NF-κBp65, Plk1, p-Elk1, COX-2, Cyclin D1, S100A6 and S100A9 was also attenuated by [6]-gingerol pre-treatment. Significant decline in p-NF-κBp65 (Figure 5.10.B.) and ERK1/2 (Figure 5.10.A.) by ~2.21 and ~1.9 fold with concomitant reduction in downstream p-Elk1, cyclin D1, COX-2, S100 A6 and S100 A9 proteins by ~1.94, ~1.43, ~1.67, ~1.42 and ~1.62 fold was recorded in the animals where [6]-gingerol-treatment was given prior to mancozeb, respectively (p<0.05) (Figure 5.10.B.).

5.4. Discussion

In the present study we determined the hyperproliferative potential of mancozeb in human skin keratinocytes and in mouse skin. Furthermore, the preventive efficacy of [6]-gingerol against mancozeb-induced neoplastic alterations in HaCaT cells was also evaluated. Although after being introduced in 1944 mancozeb has been commercially produced for almost 60 years, its potential for human carcinogenicity has been inadequately evaluated, while several animal studies have reported its carcinogenic activity (Shukla et al., 1990; Monis and Valentich, 1993; Belpoggi et al., 2002). Its carcinogenic potential for skin in Swiss albino mice has already been proved by us earlier (Shukla et al., 1990). When we exposed HaCaT cells to mancozeb in order to assess its carcinogenic potential in human skin, increases in cell proliferation (increased BrdU incorporation and PCNA expression) and neoplastic transformation (colony formation and anchorage independent growth assays) were recorded. IARC has also reported that the chances of acquiring skin and lip cancer are high among pesticide sprayers (IARC, 1991). A recent epidemiological study has shown that pesticides enhance the incidence of melanoma in farm workers by two folds (Dennis et al., 2010).

Further studies were then conducted in order to identify the affected pathways, which would significantly improve the understanding of molecular pathomechanisms of mancozeb-induced cancer and provide a fundamental platform for the creation of effective preventive strategies. In the Chapter IV of this dissertation we have illustrated
mancozeb’s ability to generate oxidative stress and its involvement in genotoxic damages inflicted by mancozeb. Thus, we initiated this pursuit of understanding the mechanism with the same line of thought and used NAC, which is a known antioxidant, in order to discern the involvement of oxidative stress in mancozeb-induced proliferation. NAC could inhibit hyperproliferation induced by mancozeb in both HaCaT cells and mouse skin, thus establishing the pivotal role of oxidative stress in this process. Corroborating our results, there are other studies where pesticides have been reported to induce oxidative stress leading to generation of free radicals and alteration in antioxidant or oxygen free-radical scavenging enzyme system (Banerjee et al., 1999; Banerjee et al., 2001; Bukowska, 2003). Involvement of oxidative stress in mancozeb-mediated neuronal damage in mesencephalic cells and DNA damage in rat cells has also been reported earlier (Calviello et al., 2006; Domico et al., 2007).

Approximately 20% of all human cancers in adults result from chronic inflammatory states which are triggered by infectious agents or exposure to other environmental aspects (De Marzo et al., 2007). Sustained cell proliferation in a milieu rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents, potentiates and/or promotes neoplastic alterations. Various evidences have shown the causal relationship of many deregulated inflammatory pathways in cancer (Wang et al., 1996; Finco et al., 1997; Martinez et al., 1997; Huang et al., 1998). Actually ROS affect metabolic processes by functioning as intracellular messenger molecules (Adler et al., 1999) and also influence signaling pathways by modulating the expression of many genes through regulation of crucial transcription factors like AP-1 and NF-κB (Goldstone et al., 1995). It has also been shown that ROS activate MAPKs, including ERK, p38, and JNK (Son et al., 2011).

In this study also proteins p-NF-κBp65, p-ERK1/2, and Plk1, that are involved in mediating the inflammation and proliferation, were found up-regulated on mancozeb exposure in both HaCaT cells and mouse skin. ERK pathway is characteristically activated by growth factors and performs a key role in cell proliferation and tumorigenesis since expression of constitutively active forms of proteins of this pathway can transform cells (Kyriakis et al., 1994; Mansour et al., 1994). Elevated ERK activation has been associated with several human cancers (Sivaraman et al., 1997; Mandell et al., 1998; Xing et al., 1999). In the present study, pharmacologic inhibition of the ERK pathway decreased mancozeb-induced neoplastic transformation in HaCaT cells as expected. Thus, the ERK pathway is clearly important for mancozeb-induced neoplastic transformation in humans. Organochlorine pesticides dieldrin, endosulfan, heptachlor,
and lindane are known to strongly activate ERK1/2 (Ledirac et al., 2005). Besides ERK, other two well characterized members of MAPK family, JNK and p38α remained unaffected when mancozeb exposure was given to HaCaT cells in this study. JNK and p38α are also sensitive to growth factors, but they are more specifically recognized as stress-responsive pathways. These pathways are implicated in inducing a homeostatic response against stress and, depending on the cellular context, are important positive or negative modulators of the apoptotic cell death program (Basu and Kolesnick, 1998; Kyriakis and Avruch, 2001). JNK is very well reported as a mediator of apoptosis (Chen and Tan, 2000; Davis, 2000; Tournier et al., 2000). A role for JNK in oncogenic transformation has also been indicated in rodent cultured cells (Raitano et al., 1995; Dickens et al., 1997; Rodrigues et al., 1997), and NIH3T3 cells (Antonyak et al., 1998; Rennefahrt et al., 2002). p38α was found to counteract oncogenic signals by inducing cell cycle arrest (Chen et al., 2000) and senescence (Haq et al., 2002; Wang et al., 2002), which led to the proposal of p38α as a tumor-suppressor protein (Bulavin and Fornace, 2004). In contrast, evidence in support of p38α having a pro-oncogenic role in cancer are also there (Rennefahrt et al., 2005). Thus, in contrast to ERK, role of JNKs and p38α in transformation has not been clearly delineated and thus a consensus has not been established for either a strictly apoptotic or antiapoptotic role for these two proteins. Our results showed that, while ERK1/2 activation is necessary, JNK and p38α are not required, for mancozeb-induced neoplastic transformation in HaCaT cells. Similarly in another study also where arsenite exposure was given, only activation of ERKs and not JNKs was reported to be required to induce cell transformation, as determined by using both dominant-negative ERK2 and JNK1 (Huang et al., 1999).

Transcription factor NF-κB, a chief regulator of many inflammatory genes, has many reports providing evidence for its involvement in cell transformation and tumorigenesis (Aggarwal et al., 2006). Pesticide methoxychlor has been reported to have inflammatory potential by inducing the expression of inducible nitric oxide synthase and proinflammatory cytokines in mouse macrophages via NF-κB, ERK, and p38 mitogen-activated protein kinases (Kim et al., 2005). Parkinsonian neurotoxin MPP⁺ exposure in SH-SY5Y neuroblastoma cells induces oxidative stress and modulates the activities of ERK and NF-κB (Halvorsen et al., 2002). In this study, we showed that the pharmacological inhibition of NF-κB by Bay11-7082 inhibited mancozeb-induced DNA synthesis, cell growth and anchorage-independent growth in HaCaT cells. We, therefore, validate that NF-κB activation is certainly required for neoplastic transformation induced by mancozeb in HaCaT cells.
Another molecule playing a key role in neoplastic progression of normal diploid cells is Plk1, which is a key controller of the G2/M checkpoint and thus is also a key determinant in the bypass of the G2/M checkpoint after genotoxic stress in normal cells thus fostering neoplastic progression (Subbaramaiah and Dannenberg, 2003; Zhang et al., 2007). Its expression is found to be up-regulated in many cancers and its overexpression can lead to dysregulated cell proliferation which is typical of the early stages of neoplastic transformation (Kastan and Bartek, 2004). In this study, specific inhibition of Plk1 by using siPlk1 could inhibit mancozeb-induced proliferation and transformation, thus we validated the involvement of this protein in mancozeb-induced neoplastic transformation in HaCaT cells.

ERK and NF-κB are important redox sensors of the cell that engage in extensive crosstalk with other signalling pathways, serving as a ‘signalling hub’ for an increasing list of growth factors, cytokines and inflammatory mediators (Handra-Luca et al., 2003; Douglas et al., 2004, Aggarwal et al., 2006). In the present study also proliferation related proteins downstream of NF-κB and ERK1/2 viz. Cyclin D1, COX-2, Elk1, S100 A6 and S100A9 proteins, were found upregulated on mancozeb exposure. ERK plays an important function in growth factor-stimulated cell-cycle progression from G0/G1 to S phase by activation of proteins like Elk1, Cyclin D1 and COX-2 (Mizuno et al., 2006). NF-κB is also known to play a very important role in carcinogenesis by regulating the expression of several proliferation associated genes including COX-2, cyclin D1, S100 A6 and S100A9 (Joo et al., 2003; Ackerman et al, 2008; Toualbi-Abed et al., 2008; Németh et al., 2009). Cyclin D1, COX-2, and Elk1 are important redox-sensitive proteins involved in inflammation associated proliferation (Ledirac et al., 2005). Cyclin D1 acts as a sensor in response to extracellular stimuli like growth factors and stress. Aberrant cyclin D1 expression has been observed early in carcinogenesis and its over-expression has been reported in several human cancers (Mizuno et al., 2006). β-Hexachlorocyclohexane (β-HCH), a contaminant of the hexachlorohexane pesticide lindane has shown breast cancer promoting activities in human breast epithelial MCF10AT1 cells and MMTV-neu mice by involving Cyclin D1 (Wong and Matsumura, 2007). Cyclin D1 has been reported to be up-regulated in molecular pathological analysis of causal mechanism behind mice hepatocarcinogenesis induced by piperonyl butoxide (PBO), a widely used synergist for pyrethrins (Muguruma et al., 2006). COX-2 is overexpressed in practically every premalignant and malignant condition (Subbaramaiah and Dannenberg, 2003). Dimethylarsinic acid (DMA) is a major metabolite of arsenic in most mammals including humans and arsenic is a well documented carcinogen. In a 2-year bioassay in male F344 rats DMA-induced urinary bladder tumors were recorded to
have up-regulated levels of COX-2 and Cyclin D1 (Wei et al., 2002). The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin), a byproduct in pesticides, is a human carcinogen. COX-2 expression was found to be involved in the development of TCDD-induced lymphomas in C57BL/10J mice (Vogel et al., 2007). Elk1 is an important protein of Ets oncogene family, also known to positively regulate COX-2 promoter activity (Ghavami et al., 2008). Pesticide DDT and its metabolites are also reported to enhance the transcriptional activity of Elk1 (Bratton et al., 2009). S100A6 and S100A9 belong to S100 family of proteins which have been linked to various cancers (Donato, 1999). Both S100A6 and S100 A9 have been reported to be regulated via redox modifications (Orre et al., 2007; Lim et al, 2009). S100A6 is a well-established marker and its level correlates with tumor invasiveness and poor prognosis in melanoma cells (Weterman et al., 1992). At molecular level, S100A9 is known to induce phosphorylation of ERK1/2 MAP kinase (Ghavami et al., 2008) and Kilanczyk et al. (2009) have suggested that binding of Calcyclin Binding Protein (CaBP) with ERK1/2 inhibits the phosphorylation of Elk1 while S100A6 competes with ERK1/2 to interact with CaBP thereby regulating Elk1 phosphorylation.

As tumor promotion is closely related with inflammation and oxidative stress, compounds that have anti-inflammatory and antioxidative activities can act as good antitumor promoters as well (Bhimani et al., 1993). With this premise we tested [6]-gingerol which is a well reported antioxidant and in chapter IV it's potential to afford a significant (p<0.05) protection against oxidative stress generated by mancozeb has been illustrated. [6]-Gingerol was reported to possess substantial antioxidant properties as determined by inhibition of phospholipid peroxidation induced by the FeCl3-ascorbate system and also of the xanthine oxidase activity (Aeschbach et al., 1994). In addition, [6]-gingerol attenuated TPA-stimulated production of superoxide generation in differentiated human promyelocytic leukemia (HL-60) cells (Rabilloud et al., 1994). In our study [6]-gingerol not only significantly (p<0.05) protected against mancozeb-induced hyperproliferative changes in HaCaT cells and mouse skin but also this protection afforded by [6]-gingerol was much better than that by NAC.

At the molecular front mancozeb-induced upregulation of p-ERK1/2, p-NF-κB, p-Elk1, S100 A6, S100A9, Plk1, COX-2 and Cyclin D1 proteins was also reduced by [6]-gingerol pre-treatment in both HaCaT cells and mouse skin. These preventive effects of [6]-gingerol on mancozeb-induced oxidative stress and up-regulation of proteins linked with inflammation-mediated proliferation, correlate with the inhibitory effect of [6]-gingerol against mancozeb-induced proliferation and transformation. Thus, this suggests that the primary effect of [6]-gingerol may be against inflammatory responses, which may thereby
result in the inhibition of mancozeb-inflicted proliferative damage. These findings are corroborated by other studies where ginger has been reported to protect against oxidative stress induced by pesticides like Malathion and lindane (Ahmed et al., 2000a; Ahmed et al., 2008). Sakr et al. (2007) reported that ginger to be able to protect against mancozeb-induced liver injury in albino rats.

Thus, it can be inferred from our study that mancozeb possesses neoplastic potential in skin keratinocyte HaCaT cells and mouse skin with the involvement of ERK1/2, NF-κB and Plk1 signal transduction pathways. Additionally, as exploration of dietary agents against pesticides'-induced damage is indubitably an upcoming prospective avenue, this data also implies that [6]-gingerol owing to its commendable antioxidant and anti-inflammatory attributes is a potent antidote against mancozeb-induced damage.