A anthraquinone derivative isolated from *Luffa acutangula* induces apoptosis in human lung cancer cell line NCI-H460 through p53 dependent pathway
Chapter-III: Induction of apoptosis through p53 dependent pathway

Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide. An alteration of tumor suppressor protein p53 is a most common genetic mechanism in lung cancer. Approximately, 50-60% of NSCLC and 90% of SCLC contain p53 mutations (Alpana et al., 2013). The tumor suppressor protein p53 play a vital role in signal transduction network, which regulates various physiological processes inducing cell cycle arrest, apoptosis or senescence (Verma et al., 2012). Recently, cell cycle arrest and apoptosis mechanisms were focused as potential molecular targets for novel anticancer drug discovery (Wang et al., 2014). From ancient period, natural products are playing an important role in the drug discovery and development and being effectively used in the treatment of various diseases including cancer (Mondal et al., 2012). Some plant derived compounds such as curcumin (Palve and Nayak, 2012), stilbene resveratrol (Kukreja et al., 2014), epigallocatechin gallate (Lecumberri et al., 2013) and silymarin (Ramasamy and Agarwal, 2008) was acting as a potent anticancer or chemotherapeutic agents which have the ability to modulate the p53 dependent apoptotic pathway or to function as a cell cycle regulator in various cancer cells (Choudhuri et al., 2007; Bhatia and Agarwal, 2001; Seo et al., 2001). The over expression of p53 induces various stresses upon the cell cycle regulation, DNA synthesis, growth factor regulation and apoptosis. The damage inducible nature of the p53 pathway is one of the mechanisms whereby the anticancer drugs or therapeutic levels of radiation induce cell death in cancer cells with a wild-type p53 pathway, whereas cells with p53 mutant form may resist damage induced apoptosis (Blaydes et al., 2000).

Anthraquinones are the most important quinone derivative of anthracene belongs to
the family of polycyclic aromatic hydrocarbons (Huang et al., 2007). Some anthraquinone derivatives such as aloe emodin, emodin and rhein have antitumor properties through the activation of p53 and p21 pathway (Kuo et al., 2002). Control of DNA replication by inducing p21 protein is one of the major functions of p53. The activation of p21 promotes cell cycle arrest by modifying the cyclin-dependent kinases (Stepulak et al., 2005). Cleia et al. (2009) reported two anthraquinone derivatives from the stem of Luffa operculata which has an anticancer potential. The effective inhibition of cell proliferation and induction of apoptosis in NSCLC by active fraction LA/II and isolation of bioactive compound 1, 8 dihydroxy-4-methylandthracene 9, 10-dione (DHMA) from fraction LA/II were discussed in the previous Chapter-II. There has been no report on the biological activity of DHMA. So in this chapter, we elucidate the anticancer potential of DHMA using NCI-H460 cell line. Further, this chapter evaluated the possible mechanisms underlying apoptosis induction, particularly focusing on the changes of reactive oxygen species (ROS), DNA fragmentation and the expression of p53, p21, NF-κB, Bax and caspase-3. The present study, suggests that DHMA exhibits the significant anticancer property through up-regulation of pro-apoptotic proteins (p53, p21, Bax and caspase-3) and down-regulation of anti-apoptotic protein (NF-κB) through the p53-dependent apoptosis in NCI-H460 cells.

**Materials and Methods**

**Materials**

DHMA used in this study was isolated from *L. acutangula*. Primary antibodies like Anti-p21, anti-p53, anti-caspase-3, anti-NF-κB, anti-bax and standard β-actin antibodies were purchased from Santa-cruz Biotechnology Inc, USA. Enhanced chemiluminescence (ECL) kit
and Cell Direct\textsuperscript{TM one}-step qRT-PCR SYBR green detection kit were purchased from GenScript USA Inc, USA. Bax, p53, ATM and GADD45A were used for mRNA expression studies and 18S rRNA house keeping gene expression kit was used for normalization.

**Cell line and culture conditions**

NCI-H460 cells were cultured as described in Chapter-I (Page No.52)

**Antiproliferative activity by MTT assay**

Materials and methods described in Chapter-I (Page No.52)

**Measurement of reactive oxygen species (ROS)**

Materials and methods described in Chapter-I (Page No.55)

**Dual-staining method (AO/EtBr) for apoptosis**

Materials and methods described in Chapter-I (Page No.56)

**DNA fragmentation assay**

Agarose gel electrophoresis was performed to examine the effect of DHMA on the induction of DNA laddering according to the method described by Ho et al. (1996). Genomic DNA was extracted from both treated and untreated cells. Briefly, NCI-H460 cells were treated with DHMA for 24 h. After incubation, cells were collected and washed with PBS buffer. The cells were centrifuged at 1000 rpm for 10 min to obtain the cell pellet. The cell pellet was suspended in 500 µl of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0), incubated at 37°C for 10 min and centrifuged at 12000 rpm for 10 min. DNA was extracted twice with phenol: chloroform (1:1), then precipitated with an ice cold ethanol and dissolved in Tris/EDTA buffer (10 mM Tris-HCL, pH 8.0 and 1 mM EDTA). DNA samples
were mixed with gel loading dye and loaded on to the wells of 1.0% agarose gel along with control. Gel was run at 60v for 1-2 h till the dye front reached one third of the gel and visualized by gel documentation system.

**Isolation of total RNA and qRT-PCR analysis**

Quantitative real time-polymerase chain reaction (qRT-PCR) for mRNA expression levels of p53, Bax, ATM and GADD45A in cell suspensions were performed using cellDirect™ one-step qRT-PCR SYBR green detection kit, in a Realplex Mastercycler (Eppendorf, Indianapolis). 18S rRNA house keeping gene expression was used for normalization.

Total RNA was extracted from treated and untreated cancer cell line with the RNeasy™ mini kit in accordance with the manufacturer’s instructions (Qiagen Company). The concentrations of extracted RNA were determined by measuring the absorbance at 260 nm and 280 nm and the quality of RNA was determined from the absorbance ratio of A260/A280 (A260/A280>1.8) and confirmed by agarose gel electrophoresis. Extracted RNA was preserved at -80°C until further use. cDNA was synthesized using 5 µg of total RNA by superscript III reverse transcriptase enzyme.

Complementary DNA was amplified in 50 µl, total volume containing SYBR green master mix and 600 nM of specific primers (Table 3.1, 3.2). RT-PCR was performed on Eppendorf master cycler and cycling conditions were as follows: For cDNA synthesis, 25°C for 10 min, 42°C for 50 min and 75°C for 15 min. For DNA amplification, 2 min denaturation step at 95°C followed by 40 cycles with 15s denaturation at 95°C, 15s primer annealing at 55°C and 20s of elongation at 68°C. For quantification, differences between treatments were analyzed by comparing mRNA levels to the control after normalization to 18S rRNA levels.
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Table 3.1. Reaction mixture for qRT-PCR analysis

<table>
<thead>
<tr>
<th>S. No</th>
<th>PCR reaction mixture</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Super script IIIRT/platinum Taq mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>2</td>
<td>2× PCR reaction mixture</td>
<td>25 µl</td>
</tr>
<tr>
<td>3</td>
<td>5 µM forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>4</td>
<td>5 µM reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>5</td>
<td>SYBR green</td>
<td>2 µl</td>
</tr>
<tr>
<td>6</td>
<td>Template RNA sample</td>
<td>2 µl</td>
</tr>
<tr>
<td>7</td>
<td>DEPEC treated water</td>
<td>18 µl</td>
</tr>
<tr>
<td></td>
<td><strong>Total volume</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>

Table 3.2. Primer sequences used in the mRNA expression of pro-apoptotic gene analysis in qRT-PCR

<table>
<thead>
<tr>
<th>S. No</th>
<th>mRNA</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p53</td>
<td>F: 5‘ CTTCGAGATGTTCCGAGAGC 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5‘ TTATGGCGGGAGGTAGACTG 3’</td>
</tr>
<tr>
<td>2</td>
<td>Bax</td>
<td>F: 5‘ CATGGAGCTGCAGAGGATGAT 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5‘ TTGCCGTCAGAAAAACATGTCA 3’</td>
</tr>
<tr>
<td>3</td>
<td>ATM</td>
<td>F: 5‘ TGGATCCAGCTATTTGTTTG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5‘ CCAAGTATGTAAACACACAGAAGTAG 3’</td>
</tr>
<tr>
<td>4</td>
<td>GADD45A</td>
<td>F: 5‘ TCAGCGACGATCAGTGTC 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5‘ CCAGCGGCAACACACCAC 3’</td>
</tr>
<tr>
<td>5</td>
<td>18S rRNA</td>
<td>F: 5‘ AGGAATTCCCAGTAAAGTGCG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5‘ GCCTCAGTAAAACCATCCAA 3’</td>
</tr>
</tbody>
</table>
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The statistical analysis of the RT-PCR results was calculated by using the ΔCt value (Ct gene of interest - Ct reporter gene). Relative gene expression was obtained by ΔΔCt methods (ΔCt sample - ΔCt calibrator), with the use of the sham-operated group as a calibrator for comparison of all gene expression levels of unknown sample. The conversion between ΔΔCt and relative gene expression levels is as follows: fold induction = 2^{ΔΔCt}, where 2^{ΔΔCt} is relative gene expression (Livak and Schmittgen, 2001).

Western blot analysis

Western blot technique is an analytical method, wherein the protein samples are separated by SDS-PAGE and electro-transferred onto PVDF (polyvinylidene fluoride) membrane. The PVDF is blocked to prevent any non-specific binding proteins. Blocked PVDF is then incubated with specific primary antibody, followed by an enzyme-linked secondary antibody. The dark bands, representing the protein, appear when chromogenic substrate is added and then the bands were quantified. In this study, western blotting analysis was carried out for p53, p21, Bax, caspase-3 and NF-κB protein expressions in NCI-H460 cells treated with DHMA according to the method of Towbin et al. (1979).

Reagents

- Acrylamide-bisacrylamide mixture: About 30 g acrylamide and 0.8 g N, N’-methylene bisacrylamide in 100 ml of double distilled water.

- Separating gel buffer (4X): About 2.25 M Tris, 0.6% sodium dodecylsulphate (SDS) was mixed with double distilled water and pH was adjusted to pH 8.8.

- Running gel buffer (5X): About 0.25 M Tris, 0.5% SDS, 1.92 M glycine 30.3 g Tris, 5 g SDS and 144.1 g glycine were dissolved in 700 ml of distilled water, pH was
adjusted to 8.3 and made one liter with distilled water. The working buffer was prepared by making a 1:5 dilution of the stock 5X buffer with double distilled water.

- Sample buffer: About 0.063 M Tris, 2% SDS, 10% sucrose, 0.01% bromophenol blue was mixed with double distilled water and pH was adjusted to pH 6.8
- 10% Ammonium persulphate (APS).
- N, N, N’N’- tetramethylethylenediamine (TEMED).
- 10% (w/v) Trichloroacetic acid (TCA): Ten gram of TCA was dissolved in 100 ml of distilled water, and stored at room temperature.

**Procedure**

NCI-H460 cells were suspended in RIPA buffer (20 mM; Tris–HCl pH 7.4; 1% NP-40; 6 mM β-mercaptopethanol; 2 mM EDTA; 0.1% SDS; 2 mM EGTA; 1 mM PMSF) containing a protease inhibitor cocktail and sonicated on ice for few seconds. The cells were centrifuged at 4°C for 10 min at 13000 rpm and the supernatant was used to determine the protein concentration by Lowry’s method (Lowry *et al.*, 1951).

**SDS-PAGE gel preparation**

Vertical slab gel electrophoresis was carried out on a Biotech slab gel electrophoresis unit. SDS-PAGE at pH 8.3 was carried out according to the method of Laemmli (1970) in a discontinuous buffer system.

The separating gel was prepared by mixing 2 ml of stock acrylamide bisacrylamide mixture, 1.5 ml separating gel buffer (0.1M Tris-HCl, pH 8.8), 0.06 ml 10 % SDS and
made to 6 ml with distilled water. 10 ml of TEMED and 30 µl of APS (10% w/v) were added, the contents mixed, degassed and poured between the assembled glass plates with edges sealed using vacuum grease. The gels were layered with 0.5 ml of distilled water and allowed to polymerize at room temperature for 30 min. The stacking gel was prepared by mixing 0.83 ml of stock acrylamide, 1.25 ml of stacking gel buffer and 50 µl of 10% SDS and making up the solution to 5 ml with distilled water. 10 ml of TEMED and 30 µl of APS (10% w/v) were added, the contents mixed and poured above the polymerized separating gel. The gel thus prepared were of the size 10.5 × 9 cm and thickness 0.8 mm.

Samples were prepared by mixing protein extract with sample buffer (2X) in the 1:1 ratio. The samples were heated in a boiling water bath for 5 min. The protein molecular weight markers were diluted 1:1 with sample buffer (2X) and boiled prior to use. Cooled samples were then loaded into the wells immersed in tank buffer and were run at constant voltage (60 V) for 6-8 hrs or until the tracking dye, bromophenol blue was just (1 cm) above the lower end of the gel plate, the power supply was turned off. The gel was lifted from the plate and removed carefully. The gel was incubated in 10% TCA for 90 min to fix the proteins.

Transfer of proteins from gel to membrane

Reagents

- Towbin buffer (for transfer): 48 mM Tris, 39 mM glycine, 1.2 mM SDS, 20% (v/v) methanol; pH 8.0
- Tris buffered saline-Tween 20 - TBST (for washing): 10 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20; pH 7.5
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After the SDS-PAGE, the stacking gel was removed and the resolving gel was used for Western blot analysis. The PVDF membrane was activated by soaking in methanol for 1-2 min. The activated PVDF membrane, resolving gel containing the protein, blotting papers and blotting sponges were soaked in cold transfer (Towbin) buffer until saturated and placed in the transfer apparatus (Bio-Rad semi-dry transfer system) filled with transfer buffer. The protein transfer was then done at 25 V for 60 min under ice-cold condition. After transfer, PVDF was blocked with a solution of 5% (w/v) non-fat milk powder in TBST for 60 min at room temperature with gentle agitation.

The PDVF was incubated with primary antibodies such Anti-p21, anti-p53, anti-caspase-3, anti-NF-κB, anti-Bax and standard β-actin antibodies overnight at 4°C with gentle agitation. After incubation with primary antibody, membrane was washed thrice with TBST with moderate shaking and then incubated in a solution of diluted secondary antibody linked with peroxidase conjugate for 2 h at room temperature with gentle agitation. Protein was detected using the western blot detection reagent kit (DAB, Genei). Similar procedure was followed for the constitutive protein, β-actin. The resultant band densities were quantified using ‘Image J’ analysis software.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) by using statistical package of social science (SPSS) version 12.0. The values are mean ± S.D for six samples in each group. p value <0.05 is considered as level of significance.
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Computational studies

Protein preparation

The crystal structure of MDM2 complex with p53 transactivation domain (PDB ID: 1YCR) was retrieved from Protein Data Bank for molecular docking studies. Prior to docking, protein was prepared using protein preparation wizard module implemented in Maestro. All water molecules were deleted and hydrogen atoms were added to the structure. The orientation of amide, hydroxyl and thiol groups and the protonation, tautomeric state of His residues were optimized. Partial atomic charges were assigned according to the OPLS-2005 force field (Kaminski et al., 2001). The structure was then subjected to impact minimization with a cut off RMSD of 0.3 Å.

Ligand preparation

The structure of DHMA was drawn using Marvin sketch tool. 3D structure conversion and minimization were performed using OPLS_2005 force field with the help of Ligprep module (LigPrep, version 2.8, 2013). About, 32 stereoisomers were generated per ligand and all possible ionization of ligand was generated at pH of 7.0. Conformer generation of each ligand was carried out using Confgen module of Maestro with default parameters. A maximum number of conformers were set as 1000 per ligand using both pre and post minimization steps that were set as 100 and 50 respectively. Each minimized conformer was filtered through a relative energy window of 10 Kcal mol⁻¹ and a minimum atom deviation of 1.0 Å. This value (10 Kcal mol⁻¹) sets an energy threshold relative to the lowest energy conformer. Conformers having higher energy than the threshold are discarded. Distances between all pairs of corresponding heavy atoms must be below 1.0 Å for two conformers to be considered identical. This criterion is applied only after
energy difference threshold and only if two conformers are within 1 Kcal mol\(^{-1}\) (Reddy et al., 2012).

**Molecular docking analysis**

Molecular docking analysis was performed using Glide version 5.7 (Glide 2011, Schrödinger, LLC, New York). Prior to the docking, the binding site was predicted using SiteMap and grid was generated around the binding site using the receptor grid generation panel. The grid-enclosing box was centered to the active sites of the corresponding 3D-structure of the receptor so as to enclose them within 3.0 Å from the centroid of amino acid residues. A scaling factor of 1.0 was set to van der Waals (vdW) radii of those receptor atoms with the partial atomic charge less than 0.25. Docking calculations were performed with XP mode, which performs systematic search of conformational, orientation and positional space of docked ligand, discarding unwanted conformations using scoring followed by energy optimization. The docking algorithm performs a series of hierarchical searches for the locations of possible ligand affinity within the binding site of MDM2-p53 complex.

**Molecular dynamics (MD) simulation**

The stability and the conformational flexibility of the MDM2-p53-DHMA complex was determined using Desmond 3.1 suite with OPLS-2005 force field (Kevin et al., 2006). The system was solvated in an orthorhombic box containing TIP3P water molecules and the salt concentration of the system was set to be 0.15 M Na\(^+\)/Cl\(^-\) for neutralizing components in a system. The distance between the box wall and protein ligand complex was set to more than 10 Å to evade direct interactions with its own periodic image (Jorgensen et al., 1996). A cut-off value of 12 Å was used for the non-bonded interactions.
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Then, the system was equilibrated with the NPT ensemble at 300 K using Nose-Hoover thermostats while the pressure was maintained by using Martina-Tobias-Klein barostat method. All production-phase MD simulations were run with a time step of 2.0 femto-seconds (fs) with far time step size of 6.0 fs using RESPA integrator for bonded and non-bonded interaction (Jatana et al., 2011). Root mean square deviation (RMSD) and root mean square fluctuation (RMSF) were analyzed to evaluate the structural changes in complex structure of MDM2-p53 receptor.

Results and Discussion

Effect of 1, 8 dihydroxy-4-methylnanthracene 9, 10-dione (DHMA) on growth of NCI-H460 cells

Phytochemicals from natural sources such as fruits, vegetables, grains and other plant have been linked to reduce the risk of various diseases, including cancer (Liu, 2004; Mondal et al., 2012). The results of the present study showed that the DHMA had a remarkable inhibitory effect on the proliferation of NCI-H460 cells. This study, first sought to determine the optimum dose for DHMA against NCI-H460. The NCI-H460 cells were treated with increasing concentration of DHMA (1, 10, 50, 100, 250 µg/ml concentration) and cell viability was assayed after 24 h treatment. These results showed that the cell survival rate was significantly reduced by dose dependent manner in NCI-H460 cells (Figure 3.1). The sensitivity of the DHMA was more prominent in the higher dosage (250 µg/ml). The results were expressed as a percentage relative to the control group. The value of 50% inhibition concentration (IC$_{50}$) of DHMA was found to be 50 µg/ml. Based on the results, the IC$_{50}$ concentration (50 µg/ml) and the highest concentration (250 µg/ml) were selected for the molecular mechanistic study.
Figure 3.1. Dose-dependent effect of DHMA on cell growth of NCI-H460 cells. The cells were treated with different concentrations (1-250 µg/ml) of DHMA for 24 h incubation and the number of cell viability was quantified by MTT assay.

Measurement of intracellular ROS by DCFH-DA method

The over production of ROS are well known mediators in the signal transduction pathway of apoptosis (Shyur et al., 2010). The increased level of ROS can induce oxidative stress, loss of cell function and finally leads to cell death (apoptosis). It can also induce lipid peroxidation and cross-linking of thiol groups in protein by which it can alter the mitochondrial membrane permeability through the opening of the mitochondrial permeability transition pore that release cytochrome-c. Then, it binds to APAF-1 (Apoptotic protease activity factor 1) and caspase-9, forming an apoptosome, which leads to the activation of caspase-3 (Rasul et al., 2013; Araujo et al., 2012). The Chapter-II suggested that the generation of ROS level by active fraction of L. acutangula was greatly increased in NCI-H460 cells. Therefore, the present study was carried out to examine whether DHMA could induce ROS production in NCI-H460 cells. As shown in Figure 3.2, the NCI-H460 cells treated with two different concentrations (50 and 250 µg/ml) of
DHMA had a higher level of ROS-associated fluorescence intensity when compared to control. The result indicates that the treatment of NCI-H460 cells with DHMA leads to the significant reduction in cell viability and its apoptotic effect was associated with increased ROS production level of ROS production in NCI-H460 cell line.

Figure 3.2 Reactive oxygen species scavenging activity of DHMA on NCI-H460, ROS was stained with DCFH-DA and generation of ROS was analyzed with fluorescence microscopy (a) Untreated cells. (b, c) NCI-H460 treated cells.

**Dual-staining method (AO/EtBr) for apoptosis**

After staining with AO/EtBr, it was observed that the viable cells with intact DNA and nucleus give uniform bright green nuclei. After DHMA treatment, early apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. The late apoptotic and necrotic cell with fragmented DNA stained orange and red. Apoptosis is an important physiological process in normal cells however, the invalid apoptosis pathway has often been one of the hallmarks for cancer and it is one of the reliable markers for the assessment of potential agents for cancer prevention (Brodska and Holoubek, 2011; Taraphdar *et al.*, 2001). Several reports have shown that the number of chemotherapeutic agents exerts anticancer property by the process of apoptosis. During apoptotic cell death, genomic DNA was cleaved into oligonucleosomal fragments due to the activation of endogenous
nuclease. This phenomenon is associated with the appearance of dense and crescent-shaped chromatin aggregates finally leads to the formation of apoptotic bodies (fragmentation of the nucleus into dense granular bodies) (Prasad and Koch, 2014) and in contrast the similar findings also observed in this study. The results from this study, clearly shows that the number of viable cells in the treated group was decreased tremendously. The untreated cells showed highly stained green fluorescence intensity compared to the treated cells, which showed a reduction in cell volume, cell shrinkage and most of the cells stained with yellow colour indicate the presence of early apoptosis in the treated cells (Figure 3.3). The microscopic examination of cell morphology of DHMA treated cells exhibited typical morphological changes, such as condensation of chromatin and formation of apoptotic bodies and chromatin marginalization. However, the number of cells stained red colour were not increased, which demonstrates that most of the cells were not undergoing necrosis and cell death occurred primarily through apoptosis.

![Figure 3.3](image)

**Figure 3.3.** Fluorescence microscopic images of AO/EtBr dual stained NCI-H460 cells with 50 and 250 µg/ml of DHMA. Untreated cells showing green colour stained cells without prominent apoptotic morphology (a). Treated cell showed characteristic feature of apoptosis, CM-chromatin marginalization; CC-chromatin condensation; BL- blebbing; CF- chromatin fragmentation, AB- apoptotic bodies (b, c).
DNA fragmentation assay

The apoptotic induction of DHMA was further confirmed by DNA fragmentation at two different concentrations (50 and 250 µg/ml). Increased levels of ROS are known to induce oxidative stress, which damages the DNA that leads to cell death. Degradation of genomic DNA is one of the key events of apoptosis and also recognized as a target of quinones. Due to its planar structure quinones is effectively involved in DNA intercalation and topoisomerase II inhibition (Muller et al., 1999). In the present study, DNA fragmentation in NCI-H460 cells after treatment with DHMA is shown in Figure 3.4. The treated cells showed a marked DNA fragmentation pattern, whereas it was not observed in untreated cells and more than four DNA fragments were visualized and the activity was found to be same at both the concentrations, whereas the DHMA treated DNA showed also most complete degradation at 250 µg/ml concentration. The patterns were consistent with the nuclear fragmentation and condensation that occurs during apoptotic cell death. The exposure of NCI-H460 cells to 50 and 250 µg/ml concentration of DHMA may directly interact with DNA by which it induces the apoptosis.

Figure 3.4. Electrophoretic separation of DNA fragments of untreated and treated NCI-H460 cells for 24 h with 50 and 250 µg/ml concentration of DHMA. Lane-1: DNA Marker. Lane-2: untreated cells; Lane-3: NCI-H460 cells treated with 50 µg/ml of DHMA; Lane-4: NCI-H460 cells treated with 250 µg/ml of DHMA.
qRT-PCR analysis of p53, Bax, ATM and GADD45A gene expression in NCI-H460 cells

In order to examine the level of mRNA expression of p53, Bax, ATM and GADD45A in NCI-H460 cells after treatment, RNA samples of both treated and untreated cells were analyzed. The activation of p53 is effectively involved in tumor suppression and cell cycle regulation. The loss of p53 expression due to mutation is a primary event in formation of various cancers including lung, breast, colon and liver cancer. It also activates another tumor suppressor gene p21 which are associated with decreasing cyclin-dependent activity in damaged cell intended to apoptosis (Haupt et al., 2003). In the present study, the mRNA levels of p53 and Bax were over-expressed under DHMA (50 µg/ml, 250 µg/ml) treatment conditions when compared to that of the untreated group (Figure 3.5 A, B). The control group did not exhibit p53 expression, but in case of DHMA treated groups the expression level of p53 was markedly increased and mRNA expression level of p21 was also increased significantly. GADD45A gene is involved in the control of cell cycle arrest, DNA repairing mechanism and apoptosis. The treatment of NCI-H460 cells with DHMA strongly induced expression of GADD45A at both concentrations. The exposure of NCI-H460 cells at a concentration of 50 µg/ml and 250 µg/ml of DHMA has resulted in the over-expression of GADD45A when compared to that of control (Figure 3.5D). This result indicates that the increased expression of GADD45A gene, which induce the genotoxic stress through p53 dependent apoptosis. ATM, a stress response gene, which responds to double strand breaks induced by anticancer drugs. Expression of ATM gene was down-regulated during DHMA (50 µg/ml) treatment when compared with control. Xing et al. (2008) reported that higher expression level of ATM has been associated significantly with increased risk of death in NSCLC patients. However, there has also been some inconsistent result in other cancer types. For example, low expression of ATM has been associated with an increased risk of death in
colorectal and breast cancers (Ye et al., 2007). Park et al. (2010) reports that the currently used EGFR inhibitor gefitinib may radiosensitize NCI-H460 cells by inhibiting ATM activity, DNA repair and thereby increasing multinucleated cell formation. In this study, the expression level of ATM was evaluated after exposure of DHMA in NCI-H460. Notably, the expression of ATM was down-regulated after treatment with DHMA at both concentrations and PTX at 10 µg/ml in NCI-H460 cells (Figure 3.5C). These results demonstrate that the p53, Bax, GADD45A gene expression could be effectively up-regulated and the expression of ATM was not involved in the processes of apoptosis in NCI-H460 cells.

**Figure 3.5.** qRT-PCR analysis of mRNA expression of p53, Bax, gene in DHMA and PTX treated NCI-H460 cells. A, B, C and D shows the relative mRNA expression pattern of p53, Bax, ATM, GADD45A respectively. Values not sharing a common marking (a, b, c) differ significantly at $p \leq 0.05$ (DMRT).
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Effect of DHMA on p53, p21 and Bax expression in NCI-H460 lung cancer cells

Western blot analysis showed increased p53, p21 and Bax expression in NCI-H460 cells after exposure of DHMA (Figure 3.6). The tumor suppressor gene p53 plays an imperative function in various types of antiproliferation and apoptosis and the induction of p21 is mostly mediated through a p53 dependent pathway. p53 regulates cell death response to various stress stimuli such as hypoxia, irradiation and chemotherapeutic agents. In the present study, the DHMA significantly induced the gene expression of the checkpoint proteins p53 and p21. The maximum expression of p53 was noticed at 250 µg/ml concentration. The inactivation of tumor suppressor protein p53 in cancer cell occurs through two general mechanisms such as inactivation of p53 functions by point mutation and partial annulment of p53 signaling pathway. MDM2 is a negative regulator of p53, which is an E3 ubiquitin ligase that targets p53 for ubiquity-dependent degradation. It also modulates p53 transcriptional activity and preventing its interaction with other general proteins involved in the transcriptional machinery (Brown et al., 2009). Blocking the interaction of MDM2 and p53 has been proposed as a potential cancer therapeutic strategy and recent studies reported that small molecules disrupting the MDM2-p53 interaction by which it restore the p53 function leading to in vivo tumor growth (Shangary et al., 2008; Vassilev et al., 2004). The activation of p21 by p53 protein and an increased level of p21 associated with decreasing cyclin-dependent activity in damaged cells destined to apoptosis (Harper et al., 1993). A marginal increase in expression of pro-apoptotic protein Bax was also observed in DHMA treated cells. Quantitative results show that the expression of pro-apoptotic proteins was significantly up-regulated in both DHMA and PTX treated cells, compared with the controls. The results imply that the DHMA induced cell death that may be mediated by p53 dependent apoptosis.
Figure 3.6. Western blot analysis shows protein expression level for p53, p21, Bax and caspase-3 after 24 h incubation with DHMA. β-actin was used as a control (A). Protein signal intensities were calculated from Image J software and the ratio of pro-apoptotic protein/β-actin were plotted (B, C). a-control, b-DHMA (50 µg/ml), c-DHMA (250 µg/ml) and d-PTX (20 µg/ml). Values not sharing a common marking (a, b, c) differ significantly at $p \leq 0.05$ (DMRT).

**Effect of DHMA on Caspase-3 and NF-κB expression in NCI-H460 lung cancer cells**

NF-κB is a transcription nuclear factor plays a major role in tumorigenesis and also implicated in cell invasion, metastasis, proliferation, and angiogenesis through regulating target gene expression. Constitutively, the over expression of NF-κB is common in a wide variety of cancers.

Therefore, the suppression of NF-κB activation is an effective measure in the prevention and treatment of cancer (Karin et al., 2006; Agarwal, 2004). The expression levels of pro-apoptotic protein like caspase-3 and anti-apoptotic function of NF-κB were determined
by western blot analysis. Caspases are acting as a crucial mediator of apoptosis. Among them, caspase-3 is an important and frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Western blot analysis shows that the caspase-3 was significantly up-regulated at both the concentrations (50 and 250 µg/ml) in NCI-H460 cells (Figure 3.7). The DHMA seemed to induce apoptosis of NCI-H460 cells through the activation of caspase-3. The DHMA inhibited the expression level of NF-κB in NCI-H460. Therefore, the inhibition of the anti-apoptotic function of NF-κB by the DHMA, may contribute to the induction of apoptosis in NCI-H460. Quantitative results show that the expression of caspase-3 was up-regulated in both DHMA and PTX, whereas the expression of NF-κB was significantly down-regulated, compared with the controls. The results indicate that DHMA might have reduced cell viability through the activation of caspase-3. These results indicate that DHMA inhibited the cell proliferation and induced apoptosis in NCI-H460 cells by increasing the ROS production, DNA fragmentation and by modulating pro-apoptotic and anti-apoptotic factors. Thereby, an outline to the mechanism of action for the anticancer effect of DHMA by triggering the apoptotic cascade leading to cell death is schematically shown in Figure 3.8.

Figure 3.7. The effect of DHMA and PTX on the protein expression of NF-κB was measured and β-actin was used as control (A). The quantification of NF-κB was analyzed by Image ‘J’ software and normalized to β-actin level (B): a-control, b-DHMA (50 µg), c-DHMA (250 µg) and d-PTX (20 µg).
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Figure 3.8. Schematic representation of the cell death mechanism by which the DHMA induced apoptosis against NCI-H460 cell line.

Molecular docking studies

Based on the *in vitro* results, a careful visual mode of binding of DHMA with MDM2-p53 was performed by computational docking. The docking study can determine the optimum conformation and binding mode of DHMA to MDM2-p53 complex to disrupt the binding of p53 with MDM2. The obtained docking energy value of -26.220 Kcal/mol and docking score value of -5.662 Kcal/mol shows that the DHMA have a higher affinity to MDM2-p53 complex. The molecular interaction between MDM2-p53 and DHMA is shown in Figure 3.9. The 1st position of OH group of DHMA interacts with carbonyl group of Trp23 by forming a 1.69 Å distance H-bond, and 8th OH group of DHMA formed hydrogen bond interaction with carbonyl group of Phe55 of MDM2 with the distance of 2.75 Å. In addition, it shows π-π stacking with Phe55 and π-cation interaction with Lys24. The hydroxyl group of
DHMA forms hydrogen bond interaction with carboxyl group of the amino acid residue Trp23. The hydrophobic residue Trp23 play a major role in MDM2-p53 interaction. Hence, the interaction of DHMA with Trp23 of p53 was responsible for the disruption of MDM2-p53 interaction. In order to explore the stable binding of DHMA into the binding pocket of MDM2-p53, MD simulation was performed using Desmond package. Several genetic studies in mouse models have shown that loss of p53 leads to the formation of tumor and the reactivation of p53 effectively suppress the proliferation of cancer cells and providing strong evidence for designing anticancer drugs which restore the function of p53 (Ventura et al., 2007; Martins et al., 2006; Xue et al., 2007). Therefore, targeting the MDM2-p53 interaction by small molecules for reactivation of p53 function has been proposed as a potential cancer therapeutic strategy for anticancer drug design and development (Fu et al., 2012). The inhibitors of p53-MDM2 interaction are attractive molecules for the treatment of wild-type p53 tumors.

Figure 3.9. Binding mode of DHMA into the active site of MDM2-p53. Hydrogen bond interactions are represented by magenta line. \(\pi-\pi\) stacking and \(\pi\)-cation interactions are highlighted by a green and red line respectively.
Molecular dynamics simulation

In order to evaluate the dynamic stability and binding affinity of the DHMA in the active site of MDM2-p53, MD simulation (20 ns) was performed using Desmond package. MDM2-p53-DHMA complex obtained from molecular docking protocol was used as an initial structure for MD simulation.

Figure 3.10. RMSD profile of MDM2-p53 complex with DHMA during the 20 ns MD simulation

Figure 3.11. RMSF profile of MDM2-p53 complex with DHMA during the 20 ns MD simulation.
As shown in Figure 3.10, the root mean square deviation (RMSD) for the backbone atoms during the simulation, indicate that the system was under dynamical condition and the conformation nature of the MDM2-p53-DHMA complex was found stable throughout the simulation within the deviation of 0.25 Å. It indicates that the system was stable and has been well equilibrated. RMSD is a crucial parameter to analyze the equilibration of MD trajectories. The hydrogen bond interaction between Phe55 and the DHMA was maintained during the whole simulation period. The flexibility of the complex was further analyzed by measuring the root mean square fluctuation (RMSF) profiles of residue and it is displayed in Figure 3.11. The high RMSF value indicates more flexibility, whereas the low RMSF value indicates limited movement of residues during the simulation. The RMSF plot has shown that some residues are more flexible during the simulation time, but there is no much fluctuation in the active site residues such as Trp53, Phe55 and Lys24. Hence, the DHMA may act as MDM2 antagonist and thereby disturb its interaction with p53 protein which leads to the detachment of p53 from MDM2 and may induce apoptosis.

Conclusion

Apoptosis is an essential mechanism through which number of chemotherapeutic agents inhibits the cell proliferation in cancer cells. The results demonstrate that the isolated DHMA induces apoptosis and ROS generation was determined through fluorescence microscopy and DNA fragmentation assay. qRT-PCR and western blot analysis was carried out to detect the expression of pro-apoptotic (p53, p21, caspase-3, Bax, GADD45A and ATM) proteins and transcription factor (NF-κB) in NCI-H460 cell line. In silico studies also performed to predict the molecular interaction of DHMA with MDM2-p53 protein. The DHMA inhibited the cell viability of NCI-H460 cells in a dose-
dependent manner. It significantly induced apoptosis and ROS generation at an effective dose of 50 µg/ml. The apoptotic activity was further confirmed through DNA fragmentation assay. DHMA significantly increased the expression of p53, p21, Bax and caspase-3 on NCI-H460 cells. The expression of NF-κB in the NCI-H460 was found to be down regulated which suggest that the mechanism of action of DHMA in lung cancer cells could possibly involve a pathway that prevents NF-κB activation. *In silico* studies demonstrate the stable interaction between the DHMA and MDM2-p53 complex, which supports the *in vitro* results. These findings suggest that DHMA induces apoptosis in NCI-H460 through p53 dependent pathway and also suggest the possibility of DHMA as a therapeutic potential for lung cancer treatment.