6. Evaluation of anticancer properties of idaein chloride on the Human Papilloma Virus (16 & 18) - positive cell lines

6.1. Introduction

Cervical cancer is the second most common cancer-type found among women that causes significant morbidity and mortality globally, though it is preventable and curable if diagnosed at an early stage. Human Papilloma Viruses (HPVs - types; 16, 18 & 31) are responsible for the occurrence 95% of cervical cancer. And the HPVs, 16 and 18 causes 75% of cervical malignant lesions (Munoz et al., 2003). The virus infects the keratinocyte in the basal layer of stratified squamous epithelium which then replicate in a differentiation – dependent manner. Expression of two early HPV genes; E6 and E7 causes cell immortalization and transformation and they specifically interfere with two important cellular tumor suppressors; p53 and pRb, as their inactivation increases the probability of cancer formation. The E6 protein binds with associated protein (AP) by forming E6AP complex by targeting p53 protein for ubiquitin-dependent degradation, whereas the E7 binds with pRb to abrogate its interaction with E2F, thereby the occurrence of up-regulation of genes for the G1-S Phase transition (Munger & Howley, 2002). In addition, several other host genes are activated including those involved in cell cycle such as Ki-67 and cyclin dependent kinase inhibitor (p16). Ki-67 is present during all the phases of cancer cell cycle and it is strictly associated with cellular proliferation, whereas in resting cells its expression is limited. The p16 is a cyclin dependent kinases inhibitor (CDKN2A) which suppresses the phosphorylation of retinoblastoma (Rb) protein (Conesa zamora, 2013). During cancer formation, p16 gets epigenetically regulated through hypermethylation of CDKN2A, which results in Phase transition of cell cycle. Another gene, Death associated protein dinase (DAPK) encodes protein which is suppressed due to aberrant methylation in promoter sequences (Gustafson et al., 2004). In mammals, the DNA methyltransferases (DNMT1, DNMT3A & DNMT3B) are methylating the DNA sequences of a gene at the specific sites. The expression of DNMT3B is associated with cancer progression (Robertzon et al., 1999).

Viral DNA integration with the host genome and expression of E6 & E7 result in malignancy of cervical cells. Antiviral therapy targeting the E6 & E7 oncoproteins results in remarkable reduction in cancer progression (Bharathi et al., 2009). The siRNA, therapeutic nucleic acids - antisense oligo DNA and ribozymes against E6/E7
mRNA are considered to be effective in controlling cancer pathogenesis (Allison et al., 2003; Dipaolo & Alvarez, 2004). Intra bodies, therapeutic antibodies and indole derivative compounds (IDCs) are capable of blocking protein – protein interaction that are the possible ways of reducing the HPV replication and capsid protein production and hence the less occurrence of HPV in cells. However, these methods have limitations like narrow specificity and high cost (Doorbar & Griffin, 2007; Soret et al., 2005; Keriel et al., 2009; Meisner et al., 2007).

The present investigation focusses on the elucidation of molecular pathways involved in cytotoxic activity of idaein chloride on HPV-16 positive CaSki and HPV-18 positive-HeLa cell lines, through induction of apoptosis by the viral oncoproteins inhibition and tumor suppressor genes activation.

6.2 Materials and Methods

6.2.1. Chemicals

Anthocyanidin - Idaein chloride, Dimethylsulfoxide (DMSO), Trypsin-EDTA, MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) and Diethyl pyrocarbonate (DEPC) were purchased from Sigma-Aldrich (USA). Dulbecco’s Modified Essential Medium (DMEM) and fetal bovine serum were bought from Invitrogen. Primary and secondary antibodies were procured from Santa Cruz Biotechnology, Inc. (USA) & Cell Signaling Technology (Beverly, MA), Protease inhibitors cock tails (aprotinin, pepstatin A, PMSF & Leupetin) were obtained from Abcam (USA) and the Polyvinylidene difluoride (PVDF) membrane from Merck Millipore (MA, USA).

6.2.2. Cell culture and idaein chloride preparation

Human cervical cancer, the HPV-18 Positive – HeLa cell line and HPV-16 Positive – Caski cell line was procured from the National Center for Cell Science, Pune (India). HeLa and CaSki cells were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-Glutamine and antibiotics (100 units/ml of penicillin and 1μg/ml of streptomycin, Sigma). Cultures were grown at humidified atmosphere of 5% CO₂ and maintained at 37°C. The idaein chloride stock solution was prepared using water and stored at -20°C until use. In each experiment, the compound was diluted freshly with DMSO (Final Conc. of DMSO was 0.1%). All
the experiments were done in triplicate for independent concentrations (0.24, 0.48, 0.96, 1.95, 3.90, 7.8, 15.6, 31.25 up to 1000 μg/ml).

6.2.3. Cell viability assay and dual staining

For cell viability assay, 1x10^6 cells/well were seeded in 96-well microtiter plate and the culture medium was replaced with fresh medium containing idaein chloride and control was maintained. At the end of the incubation, 10μl of MTT (5mg/ml) reagent was added into each well and the plates were incubated for 4 h in dark at 37°C. The resultant MTT products were dissolved in DMSO and the cell viability was calculated by measuring the optical density at 570nm by ELISA reader (Bio-Rad instruments Inc., USA). Inhibitory concentration 50 (IC_{50}) was calculated. Simultaneously, dual staining methods were used to differentiate live and dead cells with acridine orange and ethidium bromide dyes.

6.2.4. DNA fragmentation

HeLa and CaSki cells (1x10^6 cells) were plated in 30 mm culture plate, when the cells reached 70% confluency, the cells were treated with drug (IC_{50} value) and incubated for 0-12 h. After incubation, the cells were harvested by centrifugation and the collected pellet was washed twice with ice cold PBS and then the cells were lysed using 10mM Tris-Cl, EDTA and 0.2% Triton X-100 (pH 7.4) followed by Phenol: chloroform extraction and precipitation of nucleic acid. The pellet was washed with 70% ethanol, air dried and dissolved in TE buffer. Finally, the product was analyzed with DNA marker on 2% agarose gel electrophoresis and visualized under UV Transilluminator.

6.2.5. Flow cytometric analysis

To assess the effects of idaein chloride on the cell cycle, HeLa and Caski cells were treated with its IC_{50} concentration and cultured for 12h. The treated cells were harvested, washed with phosphate buffered saline (PBS), then fixed with 75% ethanol and left at 4°C overnight. After fixation, the cells were washed twice with PBS, then the cells were suspended in PBS containing 40 μg/ml propidium iodide (PI) and 0.1mg/ml RNase A followed by continuous shaking at 37°C for 30 min. Cells were analysed under flow cytometry (BD, USA) on the FL2-A detectors and the obtained data was analyzed statistically using Win MDI 2.9 software (TSRI, USA).
6.2.6. Western blotting

HeLa cells and Caski (1x10^6) were seeded onto 100-mm culture dishes and treated with idaein chloride for 12h. Then the cells were twice washed with PBS (0.01M, pH 7.2) and lysed on ice (lysis buffer containing 100μg/ml PMSF, 50mM Tris-base at pH 8.0, 150mM NaCl, 0.02% NaNO₃, 1% NP-40, 10μM aproptinin, 10 μM pepstatin A, 10 μM leupeptin). The cells were then centrifuged at 10,000 xg for 5 min and the supernatants were collected (cell protein extracts). The isolated proteins were separated on SDS-PAGE and electrophoretically transferred to PVDF membrane. Then the membrane was incubated with specific primary antibodies (Table 7) followed by HRP-anti-IgG secondary antibodies. Finally, the products were visualized through chemiluminescence ECL plus detection kit (Amersham Bioscience). The ratio of specific proteins to β-actin (internal control) was calculated.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primary Antibody</th>
<th>Company</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Apoptotic Proteins, Bax, Bcl-2, Caspase 3 &amp; 9, PARP &amp; Cytochrome</td>
<td>CST</td>
<td>1:2000</td>
</tr>
<tr>
<td>4.</td>
<td>DNMTs – 1, 3 &amp; 3A</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>5.</td>
<td>E6 &amp;E7</td>
<td>Santa Cruz</td>
<td>1:500</td>
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Table 7. Primary antibodies and their dilutions

6.2.6. Statistical analysis

All the experiments were conducted in triplicate and the statistical significance between the control and treated cell lines were analyzed by one-way ANOVA test. The significance was considered if the P value was less than 0.05.
6.3 Results

6.3.1. Inhibition of HeLa cell proliferation through apoptotic induction and inhibition of the cell cycle progression at G1 Phase

Exposure to idaein chloride showed dose dependent activity in exponentially grown HeLa and Caski cell population (Fig.15). Based on the dose response curve, the calculated half-maximal inhibitory concentration (IC\textsubscript{50}) of idaein chloride were found to be 2.579 μg/ml in HeLa and 31.64 μg/ml in CaSki cell lines. Evaluation of nuclear morphology using Acridine orange/Ethidium Bromide (AO/EtBr) indicated apoptotic cell death. It is to be noted that AO stains both live and dead cells, whereas EtBr specifically stains the DNA of the cells whose membrane integrity is lost. While the green live cells showed uniform distribution with large nuclei, the cell death occurred in idaein chloride treated cells confirming its cytotoxic effect (Figs. 16A & B). Further, the DNA fragmentation was confirmed at the dosage of 2.579 μg/ml (HeLa) and 31.64 μg/ml (Caski), the percentage of DNA breakage was found to be increased during 6 - 12 h (when compared to control), indicating time dependent increase in activity (Figs. 17A & B). In order to precisely assess the time dependent activity flow cytometric analysis was performed using propidium iodide stain at various time intervals (0, 3, 6 & 12 h). The analyses indicated an increase in death rate and specifically the idaein chloride was found to arrest the progression at G\textsubscript{1} phase which gradually increased to sub G\textsubscript{1} cell population (Figs.18A & B).
Figure 15. Cytotoxic effects of Idaein chloride on CaSki and HeLa Cells.

Figure 16A. Acridine orange and EtBr Stained CaSki Cells (C-Control; T-Treated).

Figure 16B. Acridine orange and EtBr Stained HeLa Cells (C-Control; T-Treated).

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**Figure 17A.** DNA Ladder pattern of Idaein Chloride treated Caski cells.

**Figure 17B.** DNA Ladder pattern of Idaein Chloride treated HeLa cells.
Figure 18A. Flow cytometric analytical results showing the cell cycle status of the Caski cells.
Figure 18B. Flow cytometric results showing the cell cycle status of the HeLa cells.
6.3.2. Idaein chloride treatment down-regulates the expression of HPV oncogenes- E6 & E7

The idaein chloride treated cells showed significantly reduced expression of E6 & E7 proteins during 6-12 h which in turn affected the recruitment of associated proteins p53 and pRb in both Caski and HeLa cells.

To assess the impact of E6 and E7 HPV proteins on their associated genes, the expression pattern of p53 after 3 h was assessed. Notably, decrease in E6 oncoprotein expression increases p53 gene expression. However, pRb protein levels were significantly lesser which tend to decline with increase in treatment time. These factors altogether indicates the activation of p53 induced apoptotic pathway in idaein chloride treated HPV 18(+)HeLa cells and HPV 16 (+) Caski cells. p53 downstream signaling target p21/WAF gene resulting in an increased expression after 3h of treatment (Figs.19A & 21). However, there was no significant difference in p16 & E2F gene expression levels initially but the expression level was declined after 12 h of treatment (Figs. 19A & 22) indicating idaein chloride mediated activation of p53 dependent apoptotic pathway. The observed expression patterns of apoptotic markers; BAX, Bcl-2, mitochondrial cytochrome C, cleaved caspases 3, 9 & PARP further substantiates the occurrence of p53 mediated apoptosis (Figs. 23 & 25). Furthermore, varied expression levels of cyclins and cyclin dependent kinase suggest the occurrence of apoptosis in a sequential manner (Figs. 23 & 24).

Although, the epigenetic association between p53 and DNA methylation remains unclear, some of the anthocyanins that activate p53-mediated apoptosis are known to inhibit DNA methyltransferases enzymes (DNMT1, DNMT3A & DNMT3B) that regulate DNA methylation. Idaein chloride treatment at different concentrations reduced/down regulated the DNMT1, DNMT3A and DNMT 3B genes at 12 h in HeLa cell line (Fig. 26). Notably, the expression of DNMT1, DNMT3A and DNMT3B has decreased along with p16 and an increased DAPK expression was observed after 6h (Fig.27). Similarly, Ki-67 gene was down regulated and its expression was completely absent after 6 h (Fig.24). Folate receptor gene showed reduced expression at 6 & 12 h of idaein chloride treatment (Fig.27).
Figure 19A. Immunoblotting of tumor suppressor genes and oncoproteins.
Figure 19B. Graphical representations of expressed tumor suppressor genes.

Figure 20. Graphical representations of tumor suppressor genes and oncogenes.
Figure 21. Immunoblotting expression of E6 oncoprotein, p53 and p21 proteins with its graphical representations.

Figure 22. HeLa cells expression of E7 oncoprotein and its target tumor suppressor proteins with graphical representations.
Figure 23. Expression levels of apoptotic proteins and Cycle cell proteins in CaSki cell lines.
Figure 24. Expression levels of Cycle cell proteins and proliferative marker in HeLa cell lines.
Figure 25. Expression levels of apoptotic proteins in HeLa cells and its graphical representation.

Figure 26. A Expression levels of DNMTs and its graphical representation.
6.4 Discussion

Uterine-Cervical cancer is one of the major health problems worldwide, and treatment to this cancer has been considered to be less effective due to its association with human papilloma virus. The intervention of viral infection is mainly supported by E6 & E7 oncproteins. The HPV E6 protein complexes with cellular proteins E6-AP facilitates p53 degradation via the ubiquitin dependent proteolytic system. E6 proteins of both high risk and low risk HPV types bind to p53 in vitro, but only E6 proteins of oncogenic HPV types can target p53 for degradation. E7 binds with Rb proteins and inactivate its dephosphorylation which results in cell cycle transition (Cuschieri & Wentzensen, 2008). Blocking the expression of HPV early genes by various techniques through the use of natural compounds could causes the cells to reprogram their proliferation status leading to apoptosis (Allison et al., 2003; Dipaolo & Alvarez, 2004; Doorbar & Griffin, 2007; Soret et al., 2005; Keriel et al., 2009; Meisner et al., 2007).
The present investigation shows that idaein chloride inhibits tumor cell growth and proliferation in a dose dependent manner (IC\textsubscript{50} = 31.64 & 2.579 μg/ml) in CaSki & HeLa cells. Although, it is speculated that the effect of idaein chloride could be due to free hydroxyl group in flavylum moiety, it is premature to draw any firm conclusion without examining the effects of structural modification of hydroxyl group of idaein chloride on p53 pathway genes. The effect of idaein chloride (IC\textsubscript{50} = 31.64 & 2.579 μg/ml) on p53 pathway genes at 100 fold low concentration was similar to the activity of five non-toxic anthocyanidins - cyanidin, malvidin delphinidin, pelargonidin and petunidin in human breast and gastric cancer cell lines (IC\textsubscript{50} = 200μg/ml) (Shih \textit{et al.}, 2005; Zhang \textit{et al.}, 2005). Similar to idaein chloride, the common flavonoid ‘apigenin’ inhibited the human cervical cancer proliferation at 35.89μM (IC\textsubscript{50}) through apoptotic induction by cell cycle arrest with increased SubG1 phase (Zhang \textit{et al.}, 2005). Likewise, the kahweol inhibited the tumor cell proliferation and induced apoptosis in different breast cancer cell lines at the 10-50 μM concentration (Cardenas \textit{et al.}, 2014).

The present findings delineate molecular modulation on the expression of endogenous HPV proteins, E6 & E7 which decreased after 6h of treatment of idaein chloride. As a consequence, tumor suppressor p53 re-expression has occurred and the rate of expression got increased after 6 h, with reduction in both Rb and phosphorylated Rb protein levels. The p21/WAF1 expression was p53 dependent and the expression of E2F was dependent on pRb (Tu \textit{et al.}, 2004). Presently, the p21 expression level was increased proportionately in relation to p53 restoration. But in contrast, idaein chloride treatment down regulated the E7 and its targets (pRb, E2F). The p53 dependent expression of p21/WAF had inhibited the G\textsubscript{1} phase by check point protein- cyclin dependent kinases by arresting cell cycle G\textsubscript{1} phase and its expression could be conjoined to mitochondrial BAX. BAX activated voltage dependent anion channel that resulted in release of cytochrome c and cleavage of procaspases; 9 & 3. The observed reduced expression levels of anti-apoptotic Bcl-2 suggest that apoptosis might occur through p53 mediated intrinsic mitochondrial pathway, as opined by earlier researchers (Goodwin & Dimaio, 2000; Wells \textit{et al.}, 2000).
The Ki67 and p16 are the highly disease specific marker genes employed for the detection of cervical cancer - grade as they provide specificity along with Pap smear testing (Mahata et al., 2011). Presently it was found that proliferative marker Ki-67 expression was reduced significantly (P< 0.01) and there was no evidence of protein after 12\textsuperscript{th} h of treatment. Thus, the Ki-67 protein is cell cycle phase dependent and the recorded subsequent inhibition of expression could be due to the cell cycle arrest through over expression of p53 and p21. Similar type of research carried out earlier (Van Oijen et al., 1998) showed the positive relation between Ki-67 and non-cycling cells. The presently obtained data on p16 expression patterns are similar to expression of p21. Though the expression level of p16 resembles that of p21 initially, its expression was found to be reduced later. Further, the levels of cyclins; D, E, A and their dependent kinases; 2, 4, 6 gradually decreased with increase in length of treatment time of idaein chloride. The recorded significant decreased levels of cyclins D (at 6h p<0.05), kinases 4 and 6 (p<0.01) support that cell cycle arrest that has indeed occurred at G1 phase. Earlier researchers have also opined that cyclins and CDK protein regulation could drive the cells to apoptosis (Chen et al., 2005; Zhang et al., 2005).

The p16 & DAPK genes are epigenetically regulated in cervical cancer and their expression mainly depends on the presence or absence of promoter methylation at the CpG dinucleotides. DNA methyltransferases (DNMT1, DNMT3A & DNMT3B) are family of enzymes that regulate the mammalian DNA methylation and are highly expressed in several tumors (Yang et al., 2004; Wang et al., 2013). During the present analysis, the observed elevated expression of DAPK protein (in HeLa cells) could be ascribed to the potent DNMT’s inhibitory property of idaein chloride at different concentrations. Idaein chloride inhibited the expression levels of DNMT1, DNMT3A and DNMT3B proteins at different concentrations. Similar finding was earlier reported (Wang et al., 2013), where the Black- Raspberry derived anthocyanins inhibited the DNMT1 and DNMT3B in colon cancer.

The present study showed that the idaein chloride-induced apoptosis is mediated through the intrinsic mitochondrial pathway as a consequence of p53 activation in HPV 16 & 18 positive CaSki and HeLa cells. Further-more investigations are needed for the validation of cytotoxic potential of the idaein chloride.