CHAPTER 6

Discussion
Worldwide, head and neck squamous cell carcinoma is the sixth most common cancer which poses a major cancer problem as, the treatment options available are successful usually in stages I and II only with acute and long term complications and marginal effects (Fung and Grandis 2010). Its treatment and management strategies pose a major challenge due to many factors including lack of early diagnosis augmented with poor prognosis of the disease. The present day treatment modalities including surgery, chemo- and radio-therapies for the management of different cancers including HNSCC but are met with the serious side-effects, limiting the usage of them; and furthermore, they are associated with the burden of high cost, toxicity and tumor relapse. These necessitate looking for an alternative approach which is affordable, effective, quite safe, and easily available, with less toxic effects that can control and manage growth and progression of cancer. Epidemiological studies have shown that many fruits, vegetables and phytochemicals from non-dietary sources could confer protection against a wide variety of cancers. Several phytochemicals have been isolated from fruits and vegetables as well as non-dietary sources that show strong anticancer activities against many common cancers and some of which are in clinical trials at various stages of development. Furthermore, there are several FDA approved anticancer drugs in the market that are phytochemicals and suppress a wide range of cancers. This suggests that cancer chemoprevention utilizing naturally occurring plant-derived phytochemicals is one the important and promising strategies in the prevention of various cancers. Furthermore, the non-/least-toxicity and abundancy make phytochemicals an interesting alternative against the conventional anticancer treatment modalities where dosage and cost and availability are major issues. These phytochemicals have been reported to target different
pathways normally activated in cancers like proliferative, pro-survival, angiogenic and metastatic signaling pathways.

In the present study, we evaluated the anticancer activities of AG, a main constituent of a medicinal herb *Andrographis paniculata*, that is widely used in ayurvedic formulations. It has been reported to have anti-cancer activity in several cancer cell lines and is in clinical trial for HIV, respiratory disorders and rheumatoid arthritis. However, their anti-cancer efficacy or detailed mechanisms of action in head and neck cancer is unknown. To assess the effects of AG in HNSCC, we employed various cell lines to study our objectives. The central findings in this study are that AG, exerted anti-cancer activity against three different HNSCC cell lines, FaDu, Cal-33 and UM-SCC-22B. It strongly suppressed the cell proliferation and induced cell death in them; induced cell cycle arrest, and apoptosis. The compound was found to have multiple effects on HNSCC, FaDu cells. The molecular mechanisms of their actions was found to be by modulating different pathways such as cell proliferation, cell cycle, apoptosis and it caused DNA damage by the accumulation of intracellular ROS together with decrease in DNA repair enzymes. Also this agent significantly suppressed various metastatic properties like migration and invasion in FaDu cells. Further, it should be noted that AG did not show any growth inhibitory and cell death inducing effects on normal human intestinal epithelial FHs74 Int cells like that in HNSCC at the same concentrations and treatment time points. These data suggested that AG could work as an effective and selective anticancer agent against squamous cell carcinoma that warrant further pre-clinical and clinical studies to certify their clinical usefulness.

The primary and most desired property exhibited by an anticancer agent in cancer therapy and chemoprevention is inhibition of cell growth and proliferation and induction of cell death (Singh and Agarwal 2006, Bhat and Singh 2008). In the present case, AG significantly suppressed
growth and proliferation of HNSCC cells. We observed that AG treatment decreased the total cell number as well as increased cell death in concentration- and time-dependent manner. AG (5–25 μM) treatment for 24 h reduced total cell number by 14-30% of FaDu cells. A further decrease in cell growth 16-54% and 22-64% was also observed following prolonged treatment durations of 48 and 72 h. Since, cell death induction is one of the mechanisms to slowdown the proliferation rate of cells we checked the cell death induced by AG in FaDu cells and found that the compound decreased cell death by 7-16%, 4-23% and 10-32% at 24-72 h. Similarly, AG significantly inhibited growth in Cal-33 and UM-SCC-22B and at 24 h and 48 h in a concentration and time dependent manner. In Cal -33 cell line, AG (5–25 μM) treatment for 24 h reduced total cell number by 21-47% and further decrease in cell growth 36-69% was observed at 48 h. Accordingly, AG treatment increased cell death by 7-23% and 6-36% at 24 h and 48 h respectively. Similarly, in UM-SCC-22B cell line with the same concentrations of AG there was a reduction in total cell number by 2-22% and 35-50% at 24 h and 48 h respectively. Correspondingly, AG treatment (24-48 h) increased cell death by 4-6% and 18-30%. These results indicate that AG strongly inhibits cell growth and proliferation, and induces cell death in all three HNSCC cell lines in time and dose dependent manner. This demonstrates that AG potently suppress HNSCC cell growth and proliferation, while significantly inducing cell death in them. Further AG was nontoxic and had no inhibitory effect on normal cell proliferation.

Many studies have reported a strong link between the deregulated cell cycle progression and cancer. Hence cell cycle has been identified as an important target in cancer management (Singh and Agarwal 2006). Induction of cell cycle arrest and subsequent cell death is one of the mechanisms to halt cell growth and proliferation. There are many proteins known to be involved in the tight regulation of cell cycle progression. Our data demonstrated that treatment of FaDu cells with AG induces S phase arrest (accumulation of cells in S phase by 4- 3%, 3 - 4% and 7-
16% at 5 to 25μM doses of cell cycle progression indicating that one of the mechanisms by which AG inhibits the proliferation of FaDu cells could be inhibition of cell cycle progression. Further studies demonstrated a marked decrease in the expressions of cyclin E (up to 90%) and CDK2 (up to 70%) accompanied by a strong S phase arrest. Therefore, targeting the uncontrolled cell cycle progression by AG by modulating the expression of cyclins and CDK expression could be a promising approach to halt excessive cancer cell proliferation of HNSCC cells. Cyclin-dependent kinases (CDKs) bound to their regulatory subunit cyclins, drive the events of the eukaryotic cell cycle progression (Pardee 1989). Catalytic activities of CDKs are regulated by phosphorylation and de-phosphorylation, and abundance of their respective cyclin partners and by physical association with CDK inhibitors of the Cip/p21/Kip/p27 or INK family proteins (Sherr and Roberts 1999). Here, we found that the AG -induced S phase arrest is mediated through the up regulation of cyclin-dependent kinase inhibitory proteins (Cip1/p21 and Kip1/p27). Retinoblastoma protein has been shown to play a pivotal role in regulating cell proliferation, DNA damage response, apoptosis, and differentiation. It is a tumour suppressor gene which regulates cell cycle in normal cells by interacting with transcription factor E2F which halts the production of cyclin E. So here we checked the expression of Rb and E2F it was found that AG caused down regulation of total Rb at the same time there was also significant decrease in the expression of E2F transcription factor. So we didn’t further explore the interaction of these two proteins as both were found to decrease with AG treatment. Over all, AG induces S phase arrest in HNSCC, FaDu cells by altering the levels of expression of CDK, Cyclin and CDKIs.

This explains the plausible role of these cell cycle regulatory proteins in the AG-induced S phase arrest observed in FaDu cells. Further, we examined whether AG enhances the interaction between CDKIs and CDKs and found that the AG-induced enhancement of the levels of
Cip1/p21 and their binding with CDK2. Thus, the interaction of these two proteins could have played an important role in the AG-induced S phase arrest that is observed in FaDu cells.

AG also induced G2/M arrest at 24 h and 48h in both cells Cal-33 and UM-SCC-22B and it was found to be more prominent at 24 h of treatment. Involvement of cyclin B1, Cdc25C and Cdc2 is very crucial for G2/M transition during cell cycle progression (Taylor and Stark 2001). The progression of through G2/M phase is regulated by activation of CDKs and its regulatory unit cyclins. In G2/M, the main regulator that controls the entry into mitosis is protein complex consisting of catalytic subunit Cdc2 associated with its regulatory partner cyclin B1. Cdc2 is inactive in phosphorylated from (Thr 14, Tyr 15) and it is activated after dephosphorylation by Cdc25C to form an active complex with cyclin B (Singh et al. 2005). However, inhibitory phosphorylation of Cdc25C at serine 216 residue renders it inactive and incapable for activating Cdc2 (Kawabe 2004). Consistent with these reports and the current observations, AG decreased the levels of cyclin B1, Cdc2/CDK1 and Cdc25C, in both UM-SCC-22B and Cal-33 cell lines. The current observations suggest that decreasing the effects/activities of G2/M-related proteins by AG leads to the G2/M arrest induction in UM-SCC-22B and Cal-33, HNSCC cell lines. Taken together, current observations and results indicate that CDKI-CDKs, cyclin B1-Cdc-2-Cdc25C could be the targets of AG in these cell lines leading to G2/M cell cycle arrest and consequent cell death in these cell lines.

Induction of apoptosis is linked with cell cycle arrest which is also a means to provide cells enough time to adjust with the external environmental as well as intracellular changes in the cells and undergo repair or cell death. Thus, cell cycle check-points play an important role in cell survival and proliferation. It has been reported that in many malignancies the cells become resistant to apoptosis or they do not respond to the chemo therapy (Hickman 1992). So next, we
evaluated the effect of AG on apoptosis in FaDu cells and annexin staining followed by flow cytometry showed a concentration-dependent increase in the apoptotic cells at 48 h. AG (5–25 μM) after 48 h of treatment increased apoptotic cells to 14-21% as compared to 2% in control. We further explored the extent of apoptosis and associated molecular changes following AG treatment. As expected, the increase in apoptosis was accompanied by an increase in PARP and caspase-9 cleavage. Cleavage of PARP at Asp 214 that helps in cellular disassembly is an indicator of apoptosis (Oliver et al. 1998). The role of caspase pathway in apoptosis induced by AG treatment was investigated by using pan caspase inhibitor, z-VAD-FMK that irreversibly binds to the catalytic site of caspase proteases and inhibits apoptosis. The results of this study suggested that that AG induced apoptosis in FaDu cells is mostly mediated caspase pathway; however, it also involves caspase-independent pathway/s.

The induction of apoptosis is mainly controlled by the upregulation and downregulation pro-apoptotic and anti-apoptotic protein molecules respectively. The major signal transduction pathway involved in apoptosis is that of proteins of Bcl2 family (Gross, McDonnell and Korsmeyer 1999). The proteins of the Bcl-2 family namely Bcl-2 is anti-apoptotic mitochondrial protein and known for its survival response and bax induces apoptosis. An increase in the Bax and decrease in the expression of Bcl-2 is associated with loss of membrane potential and it is the key event of induction of apoptosis characterized by reduction in ATP levels, increased permeability of mitochondrial membrane pores and influx of ions which leads to decrease in the mitochondrial membrane potential (Crompton 2000). The loss or decrease in mitochondrial membrane potential makes a cell energetically deficient and is thus lethal leading to cleavage of caspase 3 & 9 which leads to the cleavage of PARP (Kluck et al. 1997). In the light of these, in current study AG treatment decreased the anti-apoptotic protein Bcl2 and increased pro apoptotic protein Bax in a concentration and time dependent manner.
DNA damage is known to cause genomic instability leading to the formation of cancer but double stranded breaks are known to kill cancer cells (Rogakou et al. 1998). Many of the cancer drugs currently available in the market like cisplatin, doxorubicin, etoposide, camptothecin etc acts by causing double stranded breaks (DSBs). In the present study, we found that the cell death induced by AG involved DNA damage as indicated by the increased expression levels of phosphorylated H2A.X (pH2A.X) protein and formation of prominent H2A.X foci indicating DNA damage as compared to control cells.

The ATM, ataxia-telangiectasia and Rad3-related (ATR) and phosphoinositide 3-kinase-related kinases (PIKKs) are crucial for transmitting the signals to check point controls when there is a DNA damage (Helt et al. 2005). It has been reported that ATM is critical for mediating checkpoint control in cells that has been induced with ionizing radiations or double stranded breaks. Here in this study we observed that Chk1 and Chk2 remain phosphorylated at the Ser317 and Thr68, respectively during 24-48 h of treatment without any profound change in total Chk1 and Chk2 levels. For maintaining the genomic stability there are several DNA repair pathways employed by the cell like base excision repair, homologous recombination repair, mismatch repair, non-homologous end repair etc. DSBs are repaired by either NHEJ or HRR. HRR usually occurs in the S phase and G2 phases of cell cycle where, the homologous chromosome serves as a template for repair. Interestingly here, we could find that AG induced S phase cell cycle arrest in FaDu cells creating ideal state for repair by HRR. But we found that there was a decrease in the expression of Brcal and Rad51, involved in HRR pathway causing the DNA damage irreparable. Recently, it has been found that in certain cancers including head and neck cancers, the overexpression of DNA repair genes has been correlated with decreased survival rate in patients (Connell et al. 2006). Therefore, here AG induced down regulation of DNA repair
pathways along with AG induced DNA damage induction may prove to be an efficient therapy option against HNSCC cancer progression.

There are many studies that have shown that oxidative stress has anti-cancer effects (Kim et al. 2014). ROS is a normal byproduct of metabolism of oxygen and it plays an important role in cell signaling and homeostasis. However, the increased ROS from environmental stress results in DNA damage and leads to cancer development (Hyun et al. 2016). Many phytochemicals like flavanoids are known to have anti-oxidant effects (Marshall 1995). But there are some phytochemicals that are known to cause DNA damage by an increase in ROS levels. Here we found that AG induced intra cellular ROS formation, DNA damage, caused S phase cell cycle arrest leading to cell death.

Our results also showed that pre-treatment with the antioxidant NAC prevented AG induced ROS, cell number, cell death and DNA damage (Fig. 11B and 11C). These results indicate that AG-induced cell death is associated with ROS generation.

ERK and Akt are known as major survival molecules that are known to play role in the proliferation, differentiation, invasion and migration of cells (Yang et al. 2013). So we investigated the effect of AG on these survival molecules and found that AG induced up regulation of phospho ERK1/2 and down regulation of phospho-Akt in time- and concentration-dependent manner MAPK signaling pathway is also known to regulate cancer cell invasion. It is reported to regulate the transcription of MMPs by regulating activation of certain transcription factors, including members of the c-Fos and c-Jun family. In a study using sulphorane there are reports that sustained ERK1/2 activation inhibited invasion in U87MG and U373MG glioma cells (Li et al. 2014).
Moreover, there are studies and that have reported MMP-2 to be the downstream effector of ERK1/2 (Li et al. 2014). Here, in our study, as we found AG upregulated the phosphorylation of ERK1/2 and downregulated the activity and expression of MMP-2. Hence, we wanted to find whether AG induced downregulation of MMP-2 is via the activation of ERK1/2 signaling pathway. To determine it, the cells were treated with inhibitor of ERK 1/2 phosphorylation, PD98059 (50µM) and found that AG significantly reversed the downregulation of expression of MMP-2. The results verified that AG inhibited invasion in FaDu cells via upregulating ERK1/2 activation.

Epithelial-mesenchymal transition (EMT) a crucial event in tumor invasion and metastasis and is facilitated by reprogramming of epithelial cells (Wang et al. 2010). There are many evidences that that epithelial-to-mesenchymal transition (EMT), is coupled with the gain of mesenchymal phenotypes and malignant characteristics in cancer cells, that enables the cancer cells to evade apoptosis and migrate into the extracellular environment (Wu et al. 2010b). Decrease in epithelial molecule E-cadherin and an increase of mesenchymal markers namely vimentin, N cadherin etc are the most important hallmarks of EMT. In the present study we observed AG induces E-cadherin and reduces the expression of vimentin, Snail and Slug when we induced the cells with TNF-α. With TNF-α induction the, mesenchymal properties of these cells significantly got increased and AG treatment strongly inhibited the expression of vimentin and transcriptional factors Snail and Slug. These results suggest that AG treatment led the FaDu cells towards MET and controlled EMT.

In epithelial tissues, β-catenin is found to be essential for maintaining the cell layers and links the intracellular junctions and cytoskeletal proteins (Herencia et al. 2012). It is a component of the cadherin protein complex and regulates growth of epithelial cells and intracellular adhesion.
There are evidences from several studies were β-catenin nuclear localization was found to be correlated with cancer phenotype (Hanahan and Weinberg 2011). Here, we have seen that as a part of invasion process, FaDu cells had activated β-catenin scattered along the cytoplasm and nuclei. However, when the cells were treated with AG, β-catenin was localized mostly in the periphery with E-cadherin. The concomitant increase in β-catenin at the plasma membrane, coupled with lower nuclear β-catenin expression, suggests that β-catenin may be translocated away from the nucleus to the periphery of plasma membrane via β-catenin–E-cadherin complexes and is considered to be important in suppressing cancer-cell invasion and metastasis.

As a part of invasion process, FaDu cells secretes more proteolytic enzymes in their surrounding as well as activates β-catenin and transcription factors including Snail and Slug. AG treatment was able to revert this effect up to large extent. Overall, current results indicate that AG possess potential anti-metastatic properties against FaDu cell lines by modulating various metastatic related properties and thus further studies are warranted on the underlying mechanisms involved and possible applications.

**Summary and Conclusions**

The present study was taken up based upon the numerous investigations of AG in preclinical studies like treatment against HIV and acute upper respiratory tract infection (Hidalgo et al. 2013) as well as the promising results of its effect on in vitro proliferation of different tumor cell lines, representing various types of cancers, emphasizing its anti-cancer and chemopreventive potential. Moreover, the chemopreventive efficacy of AG and its associated molecular mechanisms in head and neck cancer were yet to be investigated. Hence, the study was designed to assess the anticancer effect of AG on head and neck cancer by using different HNSCC cell lines namely FaDu, Cal-33 and UM-SCC-22B and identify the specific molecular targets of AG.
that are responsible for its efficiency against head and neck cancer. Results from our study elucidated the different mode of action of AG on HNSCC and further emphasized the chemopreventive potential of AG as well as established a convincing foundation for further investigation of this compound as a lead for cancer drug development to be used singly or in combination with other approved drugs in future.

In this study, we observed that AG strongly inhibits proliferation of HNSCC FaDu, Cal-33 and UM-SCC-22B cell lines concomitant with a S phase cell cycle arrest in FaDu and G2/M arrest in both UM-SCC-22B and Cal-33 cell lines, accompanied with down regulation of cell cycle regulator proteins and increase in CDKIs. AG mediated induction of S phase arrest in FaDu cells could partly be explained by the increased interaction of CDKI-CDKs. It induced apoptosis in FaDu and UM-SCC-22B cell lines. AG exerted pleiotropic effect by modulating multiple pathways in HNSCC cells. So HNSCC, FaDu cells were used to further delineate the mode of action of AG. Apoptosis was found to be mostly caspase mediated and associated with mitochondrial membrane depolarization and an increase in Bax/Bcl-2 ratio.

We also identified that AG induced strong DNA damage and activated DNA damage check-point, ATM-Chk-1/Chk2-H2A.X pathway; however, it decreased the levels of DNA repair molecules BRCA1 and Rad51. It has been reported that the decreased survival rates and increased drug resistance in head and neck cancers is highly linked to the over expression of DNA repair genes (Furgason and Bahassi 2013). For instance, the major drawback of the current therapy available for HNSCC based on EGFR inhibitor is due to the drug resistance developed in the cells as a result of the activation of the DNA repair pathways leading to a decreased clinical outcome.
In light of our findings, AG could be used in combination with DNA damaging drugs, as AG would repress the DNA damage repair pathways that might considerably improve the clinical outcomes. Furthermore, AG caused an accumulation of intracellular ROS, which was reversed by antioxidant N-acetyl cysteine.

AG potentially suppressed metastatic attributes like invasion/migration in a concentration-dependent manner that was associated with the repression of MMP-2 and -9 activities by AG. ERK and Akt are known as major survival molecules that are known to play role in the proliferation, differentiation, invasion and migration of cells. Here we found that AG induced up regulation of phospho ERK1/2 and down regulation of phospho-Akt in time- and concentration-dependent manner. AG treatment upregulated phosphorylation of ERK1/2 which in turn resulted in the downregulation of MMP-2 expression and activity as evidenced by the treatment with PD98059, inhibitor of ERK1/2, which reversed the AG induced downregulation of MMP-2. This molecular mechanisms led to the inhibition of cell invasion. The compound was also found to strongly suppress EMT and induced the expression of epithelial marker E-cadherin and reduced the expression of vimentin, Snail and Slug. Moreover, this compound localized β-catenin mainly in the periphery with E-cadherin leading to decrease in nuclear β-catenin expression limiting cancer-cell invasion and metastasis.

These findings together provide in vitro evidence that AG suppresses migration/invasion via activating ERK1/2 leading to downregulation of MMP-2expression and reverses TNF-α induced EMT. These results may provide new insights into the molecular mechanisms of anti-metastatic properties of AG against HNSCC and offer a potential way to treat HNSCC.

In conclusion, cell cycle progression, mitogenic, pro-survival and metastatic characteristics of HNSCC are the likely targets of AG. From the studies conducted in this thesis, AG selectively
targets DNA damage and repair via enhancing intracellular ROS level, leading to cell cycle arrest and apoptotic death of HNSCC, FaDu cells. These new evidences from our studies suggest that, AG could offer potential roles in the prevention and management of HNSCC. However, these conclusions warrant further pre-clinical studies for their prospective clinical usefulness.
Comment 1: The results mentioned on page 43 of Figure 5 regarding 70% decrease of CDK2 after 48h of treatment. How the decrease is quantified should be discussed.

Answer: Fold change of CDK2 protein expression level when treated with 25μM AG is 0.3 as compared to control (value1). Therefore, taking the value of control as 100% the value of 25μM treatment would be 30%. The decrease is calculated as below

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1 = 100%
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0.3 = 0.3/1*100 = 30%
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Therefore, decrease = 100% - 30% = 70%

Comment 2: The AG treatment has no profound on FHs 74 intestinal epithelial cells. Why these cells were used as control against HNSCC should be clarified.

Answer: Firstly, FHs74 Int is of epithelial origin same as FaDu, HNSCC. Secondly, most of the anti cancer drugs act on rapidly dividing cells. Since a much higher proportion of cancer cells are undergoing active division, they are more vulnerable than most normal cells to anti-cancer drugs. However, certain normal tissues with high mitotic indices (e.g. bone marrow, spleen, thymus and intestinal epithelium) are also more susceptible/sensitive to anti-cancer drugs in the same way as rapidly dividing cancer cells. Therefore, these normal cells always get affected and killed during chemotherapy, such as the rapidly growing cells of the gut lining epithelial cells and the hair follicle cells causing diarrhea and hair loss. Hence, drugs which have minimum effects on these cells would be optimal for therapy. In light of this, normal cells derived from same tissue are not the most relevant concern for chemotherapy toxicity/ to test phytochemical toxicity as the case here. Instead, bone marrow and intestinal epithelial cells are usually more relevant and hence here, we have used the intestinal epithelial cells namely FHs74Int.