Lung cancer is among the most common and lethal forms of cancer. Non-small cell lung carcinoma (NSCLC) is prevalent and increasing in both smokers and non-smokers. Major concern with lung cancer is the late diagnosis with only 7% survival rate. Only 13% of lung cancer patients survive more than 5 years. Lethality of lung cancer pertains to its late diagnosis, low clinical response or development of drug resistance. However, the reasons behind it are not fully understood. Low survival and large number of deaths in lung cancer pertains to the lack of knowledge about the molecular events involved in the early stage of the development of lung cancer. Lack of molecular markers of detection hampered the lung cancer control. Since, the development of tumor is a multistage process, identification and understanding the signatures of normal and cancerous cells promises to provide an important insight into the aetiology of cancer that can be used for early detection, diagnosis and cancer control. Only molecular understanding about the evolution of cancer from a normal cell could help to control it. Detection of cancer at earlier stages could restrict the disease and exploitation of the reversible molecular modifications could be beneficial for its control.

Genetic modifications are irreversible whereas epigenetic modifications are reversible, thus, having more potential for the exploitation in targeting the cancer control. Epigenetic changes involve mainly DNA methylation and histone modification. Epigenetics govern cell fate and alteration in epigenetics causes different pathological disorders like development of cancer. Almost every type of human cancer, including lung cancer have some or other epigenetic defects in terms of promoter hyper methylation, histone modification or altered expression of epigenetic mediators. Gene promoter CpG methylation and histone modifications are the epigenetic signatures and have been correlated with TSG silencing in various established cancer types. In the recent past various reports have shown the potential of epigenetics in the field of cancer therapeutics and diagnosis. Certain molecular inhibitors of the epigenetic pathways are in use to control the cancer. DNMT1 inhibitor, azacytidine and HDAC1 inhibitor, vorinostat are in use for controlling CTCL or AML. Moreover, MGMT promoter methylation is used in cancer prognosis. But epigenetic is a vast field and exploring its deeper relation with cancer development could make possible to design strategies, in combination with available
measures, to prevent and may cure the cancer. Chemoprevention is also a newer field with the concept of delaying or preventing cancer remains a viable and attainable goal for the future. A number of natural or synthetic compounds including tea polyphenols, butyric acid, ω-3 PUFA, chaetocin, EGCG, curcumin have been used or under investigation as chemopreventive agent to retard or prevent the development of tumors by intervening different molecular events including epigenetics. IP6 a naturally occurring sugar phosphate, exhibiting tumor suppressing effect through its anti-angiogenic, anti-oxidant and anti-inflammatory properties and has been used in this study.

Available reports show the epigenetic modulation in tumors or in advanced cancer, but how epigenetics gets modulated during the development of lung tumors are not well known. By studying epigenetics during the development of tumor in experimental models we could evaluate their potential as a marker of detection, prognosis, and chemoprevention and target epigenetics events for therapeutic and prognostic use. This study is aimed to find out the epigenetic and associated events during the tumor development in experimental model starting from carcinogen exposure to the development of well defined tumors. We showed how epigenetic events are involved in the urethane induced mouse lung tumor development with respect to the time and their response to the anticancer agents, IP6. To accomplish our aim, objectives were as given below:

1. To evaluate the alterations in epigenetics with respect to time and duration of lung tumor development by urethane.
2. To study the expression of genes involved in methylation of DNA, transcription repression, repair, proliferation and inflammation during the development of urethane induced mouse lung tumor.
3. To evaluate the chemopreventive potential of Inositol hexaphosphate (IP6) and its effect on epigenetics in mouse lung tumor development.

In the chronic animal bioassays, we used urethane induced mouse lung tumor as experimental model and IP6 as a chemopreventive agent. Sulindac was used as a reference chemopreventive agent. Different time points were selected to follow the sequential
development of tumors and understand the sequential alterations in the molecular events which are critical to the tumor development. End points of study were from 1 to 36 weeks after urethane exposure covering the acute response of carcinogen exposure changes appeared in pre neoplastic lesions, the time when tumors started appeared in lungs and finally when tumor progresses to a well defined adenomas. Along with epigenetic changes we studied other molecular parameter related inflammation, repair, proliferation for correlating and justifying our findings.

Various tools and techniques were used in the study as per need to fulfill the objectives. Chronic animal bioassay was used to study the periodical development of tumors, histopathology was used to see the histological changes and tumor development induced by urethane after different end points. The entire was carried out in presence of IP6 in a separate set of experiments. We analyzed the molecular changes induced by urethane under all the experimental conditions in mouse lung.

Gene expression was analyzed by analyzing mRNA and protein status with RT-PCR and western blotting respectively. Protein expression and their localization in tissue were analyzed by IHC analysis. Enzyme assay was performed to analyze the functional activity of proteins in lung tissues. MS-PCR was used to analyze promoter CpG methylation where as ChIP was performed to evaluate the status of methyl histone on TSG promoter.

For evaluating alterations in epigenetics with respect to time and duration of lung tumor development by urethane, we analyzed genes involved in DNA methylation (DNMT1/3a/3b) and histone modification (HDAC1, EZH2, G9a and SUV39H1). For showing the effect of these genes we analyzed promoter CpG methylation of genes of different pathways including p16 (cell cycle), MLH1 and MGMT (DNA repair), DAPK1 (cell death) and COX-2 (inflammation). Histone modification was analyzed in term of status of methyl histones (H3K9me2 and H3K27me3) and their presence on TSGs promoters (p16 and MLH1). Presence of H3K9me2 and H3K27me3 mark on gene promoter suppress the transcription of that gene.
Expression of the genes involved in transcription repression (MBD proteins and methyl histones), repair (MLH1 and MGMT), proliferation (p16 and cyclin D1) and inflammation (Nf-kB, STAT3, IL6 and COX-2) was correlated with that of epigenetic changes. Chemopreventive potential of IP6 and the sensitivity of the molecular alterations towards IP6 were also analyzed.

Urethane induces mouse lung tumors in a time dependent manner where hyperplasia and lymphocytic infiltration present at 4 week end point and tumor arises at 12 weeks. Well defined adenomas present at 24 or 36 week end point. IP6 prevented the tumor development by preventing hyperplasia at early time points (1 or 4 weeks) and reducing the size and number of tumors in 12, 24 or 36 weeks end points. IP6 showed almost 50% reduction in urethane induced tumor development in term of number and size of tumors.

Analyzing epigenetic and molecular changes induced by urethane, we found that the key events of epigenetics got deregulated with the passage of time. DNA methyl transferases was found to be altered in a time dependent manner at mRNA and protein level showing maximum upregulation in tumor tissue at 24 or 36 week end point. Among the three DNMTs (DNMT1/3a/3b), highest upregulation was observed for DNMT1. Even DNMT1 was found to be upregulated at 1 week end point showing its importance in preneoplastic transformation. DNMT upregulation was manifested as the promoter CpG methylation of p16, MLH1, MGMT, DAPK1 and COX-2 genes. Though, the promoter CpG methylation was not much at 1week time point, but increased further at 4 weeks end point suggests its role in developing the tumors. Promoter CpG methylation of p16 and MLH1 occurred at 4 week end point and increased in 12 to 36 week end point suggesting a deregulated cell cycle and DNA repair pathway at the time of neoplastic progression and establishment of tumors. MGMT and DAPK1 promoter methylation in tumor tissue showed their importance in advancement of tumors.

HDAC1, EZH2, G9a and SUV39H1 are the histone modifying enzymes that act via remodeling chromatin state and suppressing gene transcription. Overexpression of HDAC1 and EZH2 was found to be time dependent. Upregulation of HDAC1 and EZH2 present
even at 1 week end point suggesting their role in cell proliferation and preneoplastic transformation. Whereas, the upregulation in G9a and SUV39H1 expression at 12 to 36 week end point suggested their role in developing tumors. Deregulated histone modifiers altered the status of methyl histones H3K9me2 and H3K27me3. Our western blot and IHC analysis showed increased status of H3K27me3 after 4 weeks end point and decreasing level of H3K9me2 after 12 weeks time support the deregulated histone modifiers in urethane induced development of tumors. ChIP analysis confirmed the presence of H3K9me2 and H3K27me3 mark on p16 and MLH1 promoter supporting the altered chromatin state.

In order to analyze the effect of promoter CpG methylation and histone modification, expression of TSG were analyzed. Down regulation in p16, MLH1, MGMT and DAPK1 expression at mRNA and protein level corresponded to the promoter CpG methylation and histone modification showing their role in preneoplastic transformation establishment of tumors.

Lung inflammation could facilitate the chemically induced lung tumor development and have been linked with epigenetic changes. Here we tried to answer the question if the deregulation in epigenetic pathway after urethane exposure is linked with the molecules involved in inflammation and cell cycle progression. Urethane treatment induces the upregulation of inflammatory mediators in a time dependent manner. Inflammatory mediators including STAT3, NF-kB, IL6 and COX-2 were found to be deregulated, simultaneously with epigenetic modulators, from start to finish the tumor development. NF-kB along with HDAC1 could affect the DNMT expression in neoplastic progression. Whereas, IL6 can induce the DNMT1 expression and could facilitate promoter methylation. Status of the molecules involved in the inflammation and in the cell cycle was correlated with the status of epigenetic changes taking place during the development of lung tumors.

With this study, we have provided the evidences about the status and the involvement of epigenetic and associated events during the course of the development of tumors from start to finish. In order to exploit the molecular modulations as targets for the control of tumors,
they should be responsive to the antitumor agents. The study of the development of tumors and the molecular modulations in the presence of IP6, an antitumor agent, fulfilled this aspect also. IP6 protected the urethane induced molecular changes at all the time points but the reduction at early end points of study was not significant statistically. That might be due to the early stage of the development of tumors where changes were not enough to be statistically significant. Prevention was more pronounced at the later end points due to higher degree of molecular alterations leading to the development of the tumor. IP6 protect the epigenetic changes along with inflammatory mediators induced by urethane that could be its one of the possible way of chemopreventive efficacy.

With this we came to the conclusion as given below

✓ Epigenetic changes are among the earliest changes involved in the inception and establishment of urethane induced lung tumors.

✓ Constitutive involvements of DNMTs, HDAC1 and EZH2 in urethane induced tumor development showed their tumorigenic potential and put them as attractive target for cancer control.

✓ Promoter CpG methylation and histone modification are the consequence of deregulated epigenetic modifier at early end points. Along with gene promoter CpG methylation, generation of histone methylation mark predict the advancement of urethane induced tumors.

✓ Simultaneous alteration in inflammatory mediators, STAT3, NF-κB, COX-2 and IL6, along with epigenetic regulators strengthens the molecular link between them.

✓ IP6 prevent urethane induced epigenetic and inflammatory changes that might be its possible way of exerting chemopreventive effect.
The significance of this study lies in showing the early and sequential change in epigenetics and inflammatory molecules during the development of urethane induced tumors. Alteration in epigenetics could prove to be valuable in predicting the risk of developing lung tumors. Expression of epigenetic regulators and promoter CpG methylation could be used to develop non invasive procedure of cancer diagnosis. These molecules responded to antitumor agent that showed their further exploitation in developing the newer chemopreventive targets and agents.