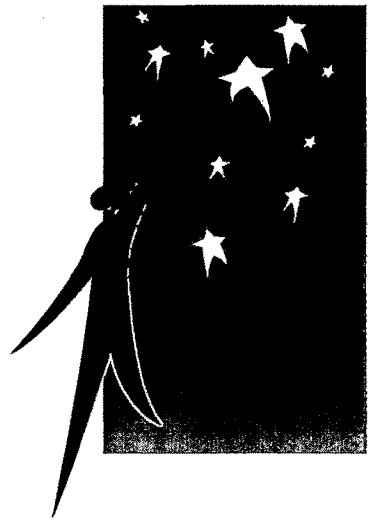
Chapter - 11

Summary & Conclusions



The tumor microenvironment is one of many areas which are studied to design new cancer therapies. More precisely, the knowledge and the understanding of the tumor microenvironment allow researchers to elaborate different therapeutic strategies, based on numerous differences compared with normal tissue including vascular abnormalities, oxygenation, perfusion, pH and metabolic states. The differences in terms of morphology of tumor vasculature and the pH will be more relevant characteristics for the design of nanocarriers as tumor targeted drug delivery systems. The passive targeting consists of the transport of nanocarriers through leaky tumor capillary fenestrations into the tumor interstitium and cells by convection or passive diffusion. In active targeting, the targeting ligands are attached at the surface of the nanocarrier for binding to appropriate receptors which over-expressed at the target site.

The clinical application of docetaxel (DTX) is limited by the poor aqueous solubility (7μg/mL), low bioavailability and high toxicity. Presently used Taxotere and Duopafei® in clinical contain high concentration of non-ionic surfactant tween-80. The adverse reactions due to either the drug itself or the solvent system have been reported in patients. In order to eliminate the tween-80-based vehicle and increase the drug solubility, alternative dosage forms have been developed, such as microparticulate lipoidal vesicles (liposomes), cyclodextrins, polymeric nanoparticles, micelles, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). Among these forms, liposomes, NLC and SLN, belong to lipid-based nanocarriers, have favourable characteristics such as: (a) improved drug dispersibility; (b) enhanced drug solubilisation; (c) enhanced drug transmembrane transport capability and (d) increased therapeutic efficacy and reduced toxicity. Therefore, to overcome conventional chemotherapy and Taxotere associated problems, in the present study, we have prepared DTX loaded liposomes composed of mixture of phospholipids (HSPC, DPPC and DPPG), as the combination of more than one lipid can increase the hydrophobic drug loading.

The docetaxel and its degradation impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel, loaded in the prepared liposomes were analysed by RP-HPLC method. The chromatographic condition includes mobile phase: acetonitrile:water (60:40, v/v); Kromacil C18 column (150×4.6mm, 5 micron, Merck); flow rate: 1mL/minute; column temperature: 30 ^{0}C; UV-visible detector; λ_{max}: 231nm.

DTX loaded liposomes were prepared, by thin film hydration technique, using combination of saturated phospholipids having high T_g at high temperature of hydration and annealing in the presence of organic acid, citric acid, added as degradation inhibitor. Based on the results obtained in preliminary experiments, cholesterol concentration (X1), hydration and annealing temperature (X2), and citric acid concentration (X3) were found to be major variables affecting % impurity formation (Y1) and % docetaxel loading (Y2). Hence, 3^3 full factorial design was applied to find the influence of these variable parameters (X1, X2, X3) on the response parameters (Y1 & Y2) and to optimize

conditions for preparing DTX liposomes with maximum drug loading and No/minimum impurity.

The liposome batch, CA-AL-25, prepared at 85 ^{0}C of hydration & annealing with cholesterol (0.0522mM) and citric acid (0.4 mg) showed evidence of docetaxel degradation (0.558±0.079%) within the Indian pharmacopoeial limit (should be less than 1%) and significant drug loading (49.64±3.12%) was optimized. Also, this batch comprises high cholesterol concentration so that we can expect decreased *in vivo* docetaxel leakage and enhanced *in vivo* liposomal circulation time. Optimized liposomal formulation described above has the pH of 5.1 (with 0.4 mg CA) with substantial drug loading and minimum 7-epidocetaxel.

At the optimized level of cholesterol (0.0522mM), temperature (85 ^{0}C), hydration and annealing time of 1.5hr, and probe sonication time of 1 minute we have tested the efficiency of other organic acids (ascorbic acid, maleic acid, and tartaric acid), as degradation inhibitor, at the same optimized concentration of citric acid (0.4mg).

We observed very less amount of impurities in liposomes prepared with tartaric acid (0.144±0.128%) as compared to citric acid (0.558±0.079%), maleic acid (0.609±0.0997%) and ascorbic acid (1.559±0.666%). This significant decrease in impurity generation is due to better control of liposomal pH by tartaric acid (pH 4.408±0.11) as compared to other acids. Hence, we used tartaric acid, compared to citric acid and other acids, as degradation inhibitor in the final liposomal formulation as it better control the impurity generation without significantly affecting the docetaxel loading (45.72±4.43%). The final optimized conventional liposomes showed mean particle size of 111±5nm (PDI: 0.266±0.101) and zeta potential of -40.2±2.4mV.

The PEGylated liposomes were prepared using DSPE-mPEG$_{2000}$ at different mol% by pre-insertion technique and analysed for the mean particle size, % DTX and impurity loading and pH. The 5 mol% PEGylated liposomes showed increased DTX loading (78.358± 2.863%) than CLs (45.72±4.59%) and other PEGylated liposomes (PLs-1mol%: 50±4.43%; PLs-3mol%: 60±2.43%). We observed increased mean particle size at 5 mol% PEGylated liposomes (119±6nm; PDI: 0.228±0.0403) as compared to CLs. The zeta potential of the prepared 5 mol% PEGylated liposomes was found to be increased (-56±2mV) as compared to conventional liposomes (-42.5±1.7mV).

The PEGylated liposomes with 5mol% DSPE-mPEG$_{2000}$ showed significantly less amount of associated serum proteins (242.085±15µg/µM) as compared to CLs (494.417±22µg/µM), PLs-1mol% (325.899±15µg/µM) and PLs-3mol% (297.194±25µg/µM) indicating the possibilities of low level of opsonisation and phagocytosis. Therefore, we can expect enhanced *in vivo* circulation time with 5mol% PEGylated liposomes as compared to conventional liposomes and other PEGylated liposomes (1mol% and 3mol%).

The optical microscopic and TEM images clearly reveal that the prepared 5 mol% PEGylated liposomes, both MLVs and SUVs, respectively were of spherical in nature. The FTIR analysis indicated no chemical interaction of docetaxel with other components of the final formulation. Hence, these excipients can be used successfully in the preparation of docetaxel loaded PEGylated liposomes. The DSC analysis indicates that the docetaxel present in amorphous and molecular state in PEGylated liposomes.

Neuropilins (NRP-1 and NRP-2) are membranous receptors capable of binding two disparate ligands, class 3 semaphorins (SEMA 3A) and vascular endothelial growth factors (VEGF-A165), and regulating two diverse systems, neuronal guidance and angiogenesis. NRP1 is expressed by a wide variety of human tumour cell lines and diverse human neoplasms, and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells. Therefore, in the present research we prepared anti-neuropilin-1 immunoliposomes by conjugating Fab' fragment of anti-neuropilin-1 antibody over PEGylated liposomes using functionalised phospholipid, DSPE-mPEG$_{2000}$-Maleimide, *via* covalent thioether linkage. The presence of sulphydryl group on reduced Fab' fragments was identified using Ellman's reagent assay. The formation of F(ab')$_2$, Fab' fragment and conjugation of Fab' fragment over liposomal surface was confirmed by SDS-PAGE analysis. The concentration of Fab' fragments attached over liposomes was determined by Bradford assay and it was found to be ~14.42±0.12µg/mL (0.0255mM of total lipid). The % Fab' fragment conjugated over liposomes was found to be 96.13%. The Fab' fragments and prepared immunoliposomes showed immunoreactivity, tested using FACS technique, similar to intact antibody.

The *in vitro* docetaxel release study revealed controlled release of docetaxel from PEGylated liposome (19.898±0.507%) and immunoliposomes (16.978±0.707%) as compared to marketed Taxotere (49.957±4.223%) after 48hr of release study. The PEGylated liposomes (4.37±0.64%) and immunoliposomes (3.163±0.48%) showed better control over the formation of 7-epimer in the release medium as compared to marketed Taxotere (14.06±2.09%) after incubation with phosphate buffer saline of pH 7.4 for 48hr. Therefore we can expect, from prepared PEGylated liposomes and immunoliposomes, less *in vivo* conversion of liposomal loaded docetaxel into 7-epimer and more passive and active targeting of loaded docetaxel to tumor tissues, and decreased 7-epimer induced systemic toxicity and tumor resistance to chemotherapy as compared to Taxotere from which about 14% of injected dose will converted into 7-epimer (as per the early reports) and cause more systemic toxicity (from our acute toxicity data) and more chance of tumor resistance to chemotherapy including docetaxel (as per the early reports). Therefore, the further *in vivo* studies are needed to ascertain these facts.

The *in vitro* suspension stability study indicates more stability of PLs containing sucrose (MPS: 188±11nm; PDI: 0.287±0.0781; zeta potential: -51±1.4mV) than PLs containing no sucrose (MPS: 480±20nm; PDI: 0.523±0.129; zeta potential: -32±4mV) after 6 month storage at 2-8 ^{0}C. The PLs containing sucrose showed about 19% decrease in DTX content as compared to PLs containing no sucrose (about 42% decrease). This indicates the need for an increase in viscosity for the stability of PLs suspension.

***In vitro* Cell Line Studies:**

All formulations resulted in concentration-dependent and time-dependent inhibition of the proliferation of A549 and B16F10 cells. The PEGylated liposomes and immunoliposomes showed more *in vitro* cytotoxicity than marketed Taxotere at the same drug concentration and exposure time, which means that for the same therapeutic effect, the drug needed for the PLs and PILs formulation could be much less than that for the Taxotere formation. Therefore, the development of the PLs and PILs thus can enhance the therapeutic effect as well as increase the maximum tolerance dose (MTD) of docetaxel. The CLs showed less cytotoxicity than Taxotere. The blank PLs had no effect on A549 and B16F10 cell growth at total lipid concentration of 15.504nM at all time points. Indeed, the differences in viability observed on cells that incubated with blank PLs and the non-treated cells were negligible.

It is very clear from the results obtained that the uptake of PLs and PILs was significantly decreased as compared to CLs in all cell lines tested. This result confirms the presence of hydrophilic PEG barrier on PLs and ILs preventing their rapid uptake by these cell lines. The conjugation of Fab' fragment of anti-neuropilin-1 antibody over PLs enhanced the cell uptake receptor mediated endocytosis. In case of A549 cell line we observed about 7-9% increased RMFI with PILs as compared to PLs at total lipid concentration 25µg for 30 and 60 minute incubation. In case of B16F10 cells, as compared to A549 cells, we observed increased uptake of PILs (about 16-20% increased RMFI) than PLs at the same lipid concentration. This might be due to more and uniform expression of neuropilin-1 receptors on B16F10 cells as compared to A549 cells. The about 45% decrease in RMFI (45% decreased uptake by K9 cells) of K9 cells treated with PILs, as compared to CLs (100%) and PLs (40% decrease), indicates that the PILs would less uptake by liver and spleen *in vivo* leading to increased circulation half time and increased accumulation at tumor tissue. The *in vitro* live uptake imaging revealed a very clear specificity of the PILs to their target. Hence, it clearly indicates their potential applications in the treatment of tumors which over express neuropilin-1 receptors.

The anti-metastatic activity of docetaxel and its PEGylated liposomes and immunoliposomes was tested by *in vitro* wound scratch assay. According to this assay the lesser the wound covered in treated cells as compared to untreated cells indicates that the drug has a good anti-migratory effect against the tested cell lines. The PILs showed very less covered wounds, more anti-migration effect, than Taxotere and PLs at the similar doses of 1nM (77.78±8.5%) and 2nM (44.66±6.6%). Similarly, against

B16F10 cells the PILs showed very less covered wounds, more anti-migration effect, than Taxotere and PLs at the similar doses of 1nM (73.04±12..94%) and 2nM (53.77±2.08%). Therefore, the significant anti-migratory effect against both the cell lines tested was observed in our study with anti-neuropilin-1 conjugated immunoliposomes and is might be due to rapid uptake of PILs *via* neuropilin-1 receptors expressed on A549 and B16F10 cells and release of loaded DTX in the cells cytosol. The released docetaxel will affect the microtubules network and thus might affect cell migration.

In the present study, we have tested the interaction of docetaxel, docetaxel loaded PEGylated liposomes, and anti-neuropilin-1 immunoliposomes with MMPs of human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10) to determine their role in tumor invasion and metastasis. The results clearly suggest that docetaxel and docetaxel loaded liposomal formulations does not affect the activities of MMP-2 and MMP-9. However, it is quite possible that they might affect other molecules involved in the invasion process.

The effect of docetaxel, docetaxel loaded liposomes on A549 and B16F10 cell apoptosis was tested using FACS technique. No significant difference in % apoptosis was observed between Taxotere, PLs and PILs after 24hr treatment against both the cell lines. However, about 8.72% and 4.66% increased apoptosis was observed with PLs and PILs, respectively after 48hr treatment as compared to Taxotere. We observed no significant difference in A549 cell apoptosis induced by PLs and PILs. Against B16F10 cell line, about 0.69% and 3.38% increased apoptosis was observed with PLs and PILs, respectively after 48hr treatment as compared to Taxotere. We observed about 2.69% increased apoptosis with PILs as compared to PLs after 48hr treatment. The qualitative apoptosis study using Ethidium bromide/Acridine orange staining method revealed that there is an increased apoptotic cells (appearing orange-reddish) with increase in time from 24hr to 48hr hours against both the cell lines. However, the effect is much more pronounced in case of human A549 cells as compared to B16F10 cells indicating A549 cells are more sensitive to treatment.

To determine the effect of Taxotere, and docetaxel loaded PLs and PILs on cell cycle, the A549 and B16F10 cells were treated with 2mL of 2nM solution of Taxotere, docetaxel loaded PLs PILs for a period of 24hr and 48hr. About 8.4% increased A549 cells in S phase were observed with PLs and PILs after 24hr treatment. No changes were observed with Taxotere treated cells. The about 10.3%, 19.37% and 26% increased A549 cells in S phase were observed after 48hr treatment with Taxotere, PLs and PILs, respectively. Thus, about 9% and 15.7% increased accumulation of cells in S phase was observed with PLs and PILs, respectively as compared to Taxotere after 48 hr treatment. The PILs showed about 6.6% increased accumulation of cells in S phase as compared t o PLs. Indicating the superiority of PILs over PLs.

Tumor Regression and Angiogenesis Study:

The targeting of chemotherapeutics to tumor blood vessels, using ligands that bind to specific molecules which over express in the tumor vasculature is a major research area. The tumor growth can be inhibited by attacking the vascular supply of the tumor. Undeniably, the host endothelial cells are believed to play a central role in tumor growth, progression, and metastasis, acting as the primary building blocks of the tumor microvasculature. As the solid tumors are angiogenesis dependent, the selective inhibition or destruction of the tumor blood vessel using either anti-angiogenic or anti-vascular treatments could trigger tumor growth inhibition, regression, and/or a state of dormancy and thereby offer a novel approach to cancer treatment. In the present study, we have developed immunoliposomes targeting to tumor endothelium and cancer cells *via* neuropilin-1 receptors which over-express on both tumor vasculature and most of the tumor cells.

Intradermal tumor model was used to study the tumor regression performance and anti-angiogenesis performance of prepared PEGylated liposomes and tumor vasculature and tumor cell targeted immunoliposomes. The totals of three doses were injected through tail vein at 2mg/kg. On 14th day the mice were sacrificed and actual tumor volumes were measured.

The treatment with PILs displayed stronger tumor inhibition than the treatment with PLs and marketed Taxotere injection. The anti-neuropilin-1 (Fab' fragments) immunoliposomes (101.937±63.647mm^3) caused suppression of tumor growth as compared to control (524.976±161.443mm^3) (p□0.05, p=0.016), PLs (404.854±101.548mm^3), and marketed Taxotere (253.354±135.892mm^3) for the data points of Day 14.

The efficient tumor suppression performance of anti-neuropilin 1 (Fab' fragment) antibody conjugated immunoliposomes can be attributed to delivery of DTX to both tumor endothelial cells (leading to vascular damage) and tumor cells *via* the over expressing neuropilin-1 receptors. Hence to confirm the vascular damage performance of PILs the same tumor model was used in which the skin containing solid tumor was collected, spreaded on whatman paper, stapled, photographed and the number of angiogenic vessels around the tumor were counted manually using the photographs. The micro-vessel density was decreased significantly around the solid tumors treated with Taxotere (p□0.05, p=0.027) and PILs (p□0.05, p=0.034) as compared to control group. This significant decrease in micro-vessel density was correlated to the strong tumor growth suppression by these two groups. The further significant suppression of tumor growth by PILs, as compared to Taxotere, would be attribute to increased blood circulation time and rapid B16 melanoma uptake of PILs *via* neuropilin 1 receptor mediated endocytosis as compared to Taxotere which would readily clear from the circulation and would remain more in the tumor interstitial space. This combined strategy of tumor blood vessel damaging and direct cancer cell killing *via* over-

expressing neuropilin 1 receptors leads to significant tumor growth suppression as compared to conventional treatment.

Although our studies have been performed in a mouse model, we expect the humanised anti-neuropilin-1monoclonal (Fab' fragments) antibody conjugated immunoliposomes can target human vasculature as well as tumor cells which over express this protein. The most of the cancer cells do over express this protein hence it will be a common target for the treatment of all types of solid tumors in patients. In conclusion, the targeting of the tumor vasculature could be the basis of a new pharmacological approach for the treatment of malignancies by taking advantage of formulations that deliver cytotoxic drugs to both blood vessels located specifically at sites of disease and to the tumor cells.

***In vitro* and *In vivo* Characterization of Docetaxel and its Impurities**

Docetaxel is obtained by semisynthesis from 10-deacetylbaccatin III, non-cytotoxic precursor extracted from the needles of the European yew, Taxus baccata. One of the principal paths of degradation is the epimerization of the hydroxyl group at position 7 which results in the formation of 7-epi-docetaxel by way of retro aldol reaction. The degradation of docetaxel can results in products which have reduced activity or are completely inactive. They also demonstrate pharmacological and toxicological profiles completely different from the active drug. Three main modifications of taxoids have been shown to occur when they are introduced in the organism: epimerization, hydrolysis, and hydroxylation. Among the three principal metabolic modifications of taxoids identified to date in the organism, hydroxylation appears to be the most important. The reversible epimerization of hydroxyl group at C-7 occurs with both paclitaxel and docetaxel. It seems to be more readily achieved in docetaxel, where it introduces additional complexity of the metabolites. The process, however, may be of minor pharmacological importance since it does not modify the cytotoxicity of the drug.

The objectives of the present research was to prepare formulations of docetaxel degradation impurities, as like marketed Taxotere and to evaluate their *in vitro* cytotoxicity behaviour against human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10), and *in vivo* acute toxicity in B16F10 pulmonary metastasis bearing mice at single dose of 40mg/kg Taxotere containing 10% of docetaxel impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel, separately. Also, the objective was to further evaluate their *in vitro* anti-metastatic character (using scratch wound assay), interaction with matrix metalloproteinases (MMP9 and MMP2), and effect on apoptosis and cell cycle.

After 3 moth storage at 2-8 ^{0}C in IMDM culture medium containing 10% foetal bovine serum, impurities remained soluble and no precipitates were observed. It is very clear from the HPLC chromatograms that, about more than 50% of 7-epidocetaxel was converted into docetaxel during the storage for 3 months. This confirms the reversible epimerisation of 7-epidocetaxel into docetaxel in the medium. Our results indicates more stability of 10-oxo-7-epidocetaxel in the medium than 7-epidocetaxel after 3 months of storage. Therefore the 10-oxo-7-epidocetaxel might remain more stable in the circulation than 7-epidocetaxel and docetaxel. Similarly, the chromatograms shows no formation of docetaxel and other impurities in phosphate buffer saline (pH 4 adjusted with citric acid) indicating more stability of these impurities at pH 4 during storage. The impurities remained soluble and no precipitates were observed after 3 months of storage. Therefore the impurities were stored in phosphate buffer saline (pH 4) throughout the study.

***In vitro* Cell Line Studies:**

The *in vitro* cytotoxicity study results revealed that the 7-epimer showed more cytotoxicity against A549 cells than Taxotere after 24hr treatment but remain less cytotoxic than Taxotere after 48 and 72hr treatment. The 10-oxo-7-epidocetaxel showed less cytotoxicity than both Taxotere and 7-epimer after 24hr, 48hr, and 72hr of treatment. Therefore, the 10-oxo-7-epidocetaxel was found less cytotoxic against A549 cell line as compared to 7-epidocetaxel and marketed Taxotere. In case of B16F10 cells the IC_{50} values were shifted to higher values after 24hr, 48hr and 72hr of treatment as compared to A549 cells indicating B16F10 melanoma cells are less sensitive than A549 cells. Against both the cell lines the marketed Taxotere showed low IC_{50} values (more cytotoxicity) than 7-epidocetaxel and 10-oxo-7-epidocetaxel.

The anti-metastatic activity of docetaxel and its impurities was tested by *in vitro* wound scratch assay. Surprisingly, we have observed about 1.46 fold (17.5% increased activity) and 1.92 fold (36.5% increased activity) increased *in vitro* anti-metastatic activity against A549 cells with 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively as compared to Marketed Taxotere. Against B16F10 cells, the 7-epidocetaxel showed 17% decreased anti-metastatic activity than marketed Taxotere but the 10-oxo-7-epidocetaxel showed about 16% increased anti-metastatic activity than marketed Taxotere. The 10-oxo-7-epidocetaxel showed significantly increased anti-metastatic activity against both the cell lines tested as compared to Taxotere. Our study truly identified a novel role of impurities specially 10-oxo-7-epidocetaxel as a potent inhibitor of cell migration. This is a preliminary observation being noticed as per now but the further investigations are needed to address the potential of 10-oxo-7-epidocetaxel in this era of cancer therapeutics.

In the present study, we have tested the interaction of docetaxel, and its impurities with MMPs of human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10) to determine their role in tumor invasion and metastasis. The results clearly suggest that docetaxel and its impurities do not affect the activities of MMP-2 and MMP-9. However, it is quite possible that they might affect other molecules involved in the invasion process.

The effect of docetaxel, and its impurities on A549 and B16F10 cell apoptosis was tested using FACS technique. The increased % apoptosis of A549 cells and B16F10 cells was observed with increase in concentration of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel from 2nM to 25nM. About 4.86 and 10.08 fold increased apoptosis of A549 cells was observed with Taxotere as compared to 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively at 2nM concentration and at 24hr of treatment. This indicates Taxotere is highly cytotoxic at low concentration as compared to docetaxel impurities tested. Against B16F10 cells, the Taxotere and impurities showed almost similar % apoptosis at 2nM concentration after 24hr and 48hr treatment. The 10-oxo-7-epidocetaxel caused % apoptosis similar to 7-epimer but caused 5.97% and 7.77% less

apoptosis at 10nM and 25nM, respectively than Taxotere after 24hr of treatment. The 10-oxo-7-epidocetaxel caused apoptosis similar to Taxotere and about 5.52% increased apoptosis than 7-epidocetaxel at 25nM and after 48hr of treatment. We observed decreased B16F10 cell apoptosis as compare to A549 cells at almost all concentrations tested and after 24hr and 48hr treatments. This clearly indicates that the B16F10 cells are less sensitive to Taxotere and impurities as compared to A549 cells.

We observed that the docetaxel and its impurities blocked the A549 cell cycle at both S phase and G2-M phase based on their concentration and treatment time. At 24hr treatment with 10nM, we observed that the Taxotere caused more accumulation of cells at S phase, whereas the 10-oxo-docetaxel blocked more at G2-M phase and vice versa at 24hr treatment with 25nM concentration. The results indicate that the B16F10 cells are less sensitive at both 2nM and 10nM as compared to A549 cells. Although, no accumulation of cells at S phase was observed with Taxotere and 7EDTX at all concentrations and treatment times as compared to A549 cells, the 10-oxo-7-epidocetaxel caused about 13.14% increased accumulation of B16F10 cells at S phase after 48hr treatment with 25nM concentration.

***In-vivo* Acute Toxicity Study:**

The B16F10 melanoma lung metastasis mice model was used to compare the single *i.v.* dose acute toxicity of Taxotere for injection alone and with 10% 7-epidocetaxel and 10-oxo-7-epidocetaxel impurities. The weight measurements of the mice were performed during the experiments to evaluate the toxicity.

As compared to control group about 2.09, 1.73, 1.44, 1.98, and 4.05 fold increase in mean group weight loss was observed with Taxotere treated group on 2, 4, 6, 8, and 10th day, respectively. This increased mean group weight loss can be correlated to the toxicity of Taxotere injection. The inclusion of 10% 7-epidocetaxel in Taxotere injection results in 1.56, 1.5, 1.35, and 1.12 fold increase in mean group weight as compared to Taxotere containing no 7-epidocetaxel at 2, 4, 6 and 8 day of drug administration, respectively. This extra loss of body weight indicates the more toxicity of 7-epimer as compared to docetaxel. Therefore, we can say that the total toxicity that occurs during chemotherapy with Taxotere is because of both docetaxel and 7-epimer formed in blood after Taxotere infusion. When we compared the toxicity of Taxotere with 10% 10-oxo-7-epidocetaxel treated group with Taxotere treated group, we observed almost similar toxicity at 4th and 6th day, but at 8 and 10th day about 2.68% and 10.4% decreased body weight loss was observed as compared to Taxotere treated group. Surprisingly, we observed the less toxicity of Taxotere with 10% 10-oxo-7-epidocetaxel as compared to Taxotere alone. The inclusion of 10% 10-oxo-7-epidocetaxel surprisingly decreased the total toxicity of Taxotere. No therapeutic effect (decreased colony formation) was observed with Taxotere and Taxotere with 10% 7-epimer treated groups at 40mg/kg single dose as compared to untreated control group. Surprisingly, the decreased colony

formation was also observed with Taxotere with 10% 10-oxo-7-epidocetaxel treated group at the same single dose (40mg/kg) as compared to all other groups.

***In vivo* Therapeutic Study:**

We observed surprisingly, in acute toxicity study, the decreased % average body weight loss and decreased number of colonies with Taxotere containing 10% 10-oxo-7-epidocetaxel treated group as compared to Taxotere and Taxotere with 10% 7-epimer treated group. Therefore, the further evaluation of the therapeutic effectiveness of the docetaxel impurity, 10-oxo-7-epidocetaxel, alone in mice pulmonary metastatic model was performed to confirm our previous results. We tested the therapeutic effectiveness of 10-oxo-7-epidocetaxel at single dose of 20mg/kg (400µg/mouse), half of the Taxotere tested dose in acute toxicity study.

The 10-oxo-7-epidocetaxel treated group showed no decrease in % body weight, instead the % mean group weight keep increased throughout the experiment and observed about 4% increased mean group weight at the end of the experiment. Therefore we can conclude that the injected 10-oxo-7-epidocetaxel caused no toxicity (reduction in body weight) at the tested dose of 20mg/kg.

The numbers of surface metastatic nodule observed were found significantly less with 10-oxo-7-epidocetaxel treated group (107±49) (***p□0.0001) as compared to control group (348±56). Therefore the 10-oxo-7-epidocetaxel showed very significant and promising results (significantly decreased toxicity with increased therapeutic effect) than the marketed Taxotere (lungs were found completely black even at 40mg/kg dose) and further studies (including clinical studies) are needed to make use 10-oxo-7-epidocetaxel as potent anti-cancer agent.