8.1. Summary

The current chemotherapy for lung cancer has limitation of being non-selective and result in toxicity. The present research work was aimed to develop stable antibody conjugated drug nanoparticles using suitable antibody as an active targeting moiety to selectively take this drug to the tumor site. Docetaxel in nanoparticulate form and antibody conjugated nanoparticles were used to target non-small cell lung cancer cells to achieve selective uptake of nanoparticulate formulations and hence to avoid non selective distribution of Docetaxel in other tissues and synergistic effect to kill or stop the progression of cancerous tissue growth. The overall hypothesis is that nanoparticulate drug carriers anchoring appropriate ligands can be targeted to tumor cells.

The UV-visible spectrum obtained by scanning the 10µg/ml of docetaxel recorded between 200nm to 400nm. It was observed that docetaxel shows the characteristic peak at 229nm and thus it was selected as the analytical wavelength. Analytical method for docetaxel was developed and validated by UV spectrophotometrically in acetonitrile. The high correlation coefficient in the above solvent indicated that absorbance and concentration of the drug was linearly related. Beer's law was found to be obeyed in the range of 5 to 45µg/ml for docetaxel in acetonitrile.

As a part of method validation, there is negligible difference in the absorbance values of the fresh and the stored solutions indicating that docetaxel is stable over the period of analysis. The accuracy of the developed method of docetaxel in acetonitrile was found to close to 100%, between 98.00 to 102.00%. In precision study, %RSD values were not more than 2.0% in all the cases. RSD values found for the analytical methods were well within the acceptable range indicating that these methods have excellent precision. The LoD and LoQ for the assay of Docetaxel using Acetonitrile is 0.175 µg/ml and 0.195 µg/ml respectively indicating that the method is sensitive. It is also selective as evidenced by the noninterference of the excipients used in the formulation of the injection. The developed spectrophotometric methods for determination of Docetaxel are simple, specific, accurate, precise, rapid and economical which indicates its adequacy for routine pharmaceutical analysis. It is concluded that the developed spectrophotometric method can be successfully utilized for the routine estimation of Docetaxel.

The Emulsification Solvent Evaporation method was employed for the preparation of Docetaxel PLGA Nanoparticles. Different solvents were tried in Emulsification Solvent Evaporation method to get particle size and drug entrapment optimum. The size if nanoparticles was reduced using acetone as solvent and PVA as a surfactant for emulsification. Optimization of the Docetaxel PLGA Nanoparticles were done on the basis of effective drug entrapment and desired particle size. Diffetrent compositions were tried and optimized for maximum drug entrapment within minimum amount of polymer.

Docetaxel Nanoparticles were characterised for particle size and zeta potential and assay, drug loading, entrapment of docetaxel. Product and Process parameters such as organic solvent, ratio of solvent to water, concentration of drug and polymer were optimized to obtain desired formulation characteristics. Docetaxel PLGA Nanoparticles formulations were optimized using factorial design by varying the Drug concentration (0.5, 1 and 2 mg/mL), polymer concentration (5, 10 and 15mg/mL) and ratio of solvent to water (0.1, 0.3 and 0.5) at three different levels such as low (-1) middle (0) and high (1) for higher drug content and lower particle size by keeping all other process same.

The increase in drug concentration shows increase in drug loading and mean particle size. At 2mg/mL drug level, we observed maximum Docetaxel loading and optimum particle size. With increase in Drug concentration beyond this level, we observed similar amount of drug loading but increased mean particle size than drug concentration 2 mg/mL. Hence, we considered drug concentration of 2 mg/mL as optimal condition.

The increase in polymer concentration shows increase in mean particle size and show minimum effect in drug loading. At 15 mg/mL drug level, we observed minimum Docetaxel loading.

The increase in ratio of solvent to water show drug loading higher at ratio of 0.5 and very negligible effect on particle size and PDI. Docetaxel Nanoparticles formulation having favorable property was subjected for further antibody conjugation for active targeting.

Optimal formulation was further conjugated by incorporation of tumor cells targeting antobody (10 µg, 20 µg and 500 µg) Cetuximab. The activation pH, reaction temperature, activation time,

amount of activating agents (EDC/NHS) and Cetuximab to NPs ratio were optimized to achieve minimum particle size and maximum conjugation efficiency.

Antibody-conjugation is generally used as a carboxyl activating agent in the pH range of 4.0-6.0 and hence a low conjugation efficiency was observed at pH 7 followed by at pH 6. Hence, taking into account the conjugation efficiency and %EE, the activation pH was optimized as 5 for nanoparticle activation followed by antibody conjugation effected at pH 7.4 to avoid denaturation of protein at lower pH. It was observed that the activation pH did not significantly affect the mean particle size of antibody-conjugated nanoparticles. At a reaction temperature of 25°C both the conjugation efficiency and %EE were observed to be the lowest which may be attributed to low glass transition temperature of PLGA resulting in increased drug leaching from nanoparticles and availability of less surface carboxyl group to effect conjugation. The same may explain for a low conjugation efficiency and %EE at 20°C. In addition, carbodiimide coupling has been reported to be efficient at room temperature. While both conjugation efficiency and %EE were observed to be high at a temperature of 15°C and hence, was optimized as reaction temperature. It was observed that increasing the concentration of activating agent from 6.0 to 9.0 μM there was an increase in conjugation efficiency from 27.65 ± 2.2% to 39.77 ± 3.4% with no appreciable effect on mean particle size or %EE of antibody conjugated nanoparticles. While, there was no significant increase in antibody conjugation efficiencies of nanoparticles with increase in EDC/NHS concentration from 6.0 to 9.0 μM μM indicating nanoparticle surface saturation with unavailability of surface carboxyl groups for more antibody attachment. The conjugation efficiency of antibody to nanoparticles was observed to be significantly low with a reaction time of 1 hr (half an hour each for activation and conjugation) with no significant effect on particle size and %EE indicating the time to be insufficient to achieve maximum antibody conjugation. However, there was no significant increase in antibody conjugation, mean particle size and %EE with increase in reaction time from 2 to 3 hr. Thus, the reaction time was standardized as 2 hr.

For all the three drug nanoparticles, with increase in the amount of antibody, the conjugation efficiency increased with no significant increase in particle size. This is because by increasing the antibody concentration from 10μg to 500μg no surface saturation was observed for PLGA nanoparticles with respect to the antibody attached as it has not been used in molar ratios.

Various types of cryoprotectants (Sucrose, Trehalose and Mannitol) are used at different ratio to optimize the lyophilization and to preserve particle size during freeze drying. The lyophilized formulations were tested for particle size, zeta potential and physical appearance. With the use of sucrose as cryoprotectant, the cake formed after lyophilization was found to be of condensed and collapsed structure. Hence, the redispersibility of nanoparticles was poor and was only possible with sonication. The Sf/Si values were 3.26, 2.53 and 2.27 with 1:1, 1:2 and 1:3 nanoparticle to sucrose respectively. The increase in the particle size could have been due to the cohesive nature of the cryoprotectant. Further, it was observed that the lyophilized nanoparticles with sucrose had tendency to absorb moisture quickly. While, with mannitol the lyophilized product was fluffy and snow like voluminous cake. Also, the nanoparticle formulation showed free flowing. However, the redispersibility of nanoparticles was difficult and possible only after vigorous shaking. The particle size, recorded in table 4.16, increased significantly after lyophilization than the initial. The Sf/Si values were 2.32, 2.03 and 1.18 with 1:1, 1:2 and 1:3 nanoparticle to mannitol respectively. This may be due to the low solubility of mannitol in water (0.18 parts of mannitol soluble in 1 part of water). With trehalose also, the lyophilized nanoparticles formed fluffy and snow like voluminous cake. With trehalose as cryoprotectant, the lyophilized nanoparticles were redispersed easily and the increase in particle size was not significant as indicated by Sf/Si values 1.7, 1.36, and 1.08 for 1:1, 1:2 and 1:3 nanoparticle to trehalose respectively. The redispersion of the nanoparticles depends on the hydrophilicity of the surface. The easy redispersibility could be probably due to the higher solubility of trehalose in water (0.7 parts in 1 part of water). The cryoprotective effect may be attributed to the ability of trehalose to form a glassy amorphous matrix around the particles, preventing the particles from sticking together during removal of water. In addition, the property of the Tyndall effect observed with nanoparticles was retained after redispersion of the nanoparticles lyophilized using trehalose. Therefore, trehalose at a ratio of 1:4 (nanoparticles: trehalose) was used as cryoprotectant for lyophilization of optimized batch of nanoparticles for further studies.

Prepared drug nanoparticles formulations were characterised for in vitro cell line studies. The cytotoxicity of Docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles were determined using 3-(4, 5-dimethylthiazole-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay. Cells were treated separately with free drug, Docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles at varying different concentrations in DMEM media containing 10%

FBS and antibiotics. It was seen that all formulations resulted in concentration dependent inhibition of the proliferation of A549. The lowest cell viability, i.e. the highest cell mortality, appeared at the highest concentration of the Docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles, which proves the controlled and sustained efficacy of the nanoparticulate formulation. Furthermore, the nanoparticulate formulations prevent the toxic effect of the drug applied at high concentration of drugs and thus can increase the maximum tolerance dose (MTD). It is clear from the results that the docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles demonstrated higher cytotoxicity than the free drug formulation at the same drug concentration and exposure time, which means that for the same therapeutic effect, the drug needed for Docetaxel Nanoparticles and Cetuximab Conjugated Docetaxel Nanoparticles formulation could be much less than that for the free drug. Therefore, the development of the Docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles thus can enhance the therapeutic effect of Docetaxel.

For cellular uptake studies, 6-Coumarin encapsulated cetuximab conjugated Docetaxel nanoparticles was used. Flow cytometry was utilized for quantitative cell uptake to determine the mean fluorescent intensity while qualitative intracellular accumulation was determined using confocal microscopy.

Cellular internalization of 6-Coumarin encapsulated cetuximab conjugated Docetaxel nanoparticles in A549 cells was monitored by confocal microscopy. Cells were transfected with nanoparticulate formulations containing 6-Coumarin. Cells were also stained with nucleus staining dye DAPI and proceeded for confocal microscopy using confocal laser scanning microscope. Confocal microscopy also showed that cetuximab conjugation helps to enhance the cellular localization in both cell lines.

In vivo efficacy of developed nanaoparticulate formulations were evaluated SCID mice. In vivo efficacy data show that docetaxel nanoparticles exert a strong antitumor activity over free docetaxel in A549 NSCLC subcutaneous xenograft model. When conjugated with cetuximab with docetaxel nanoparticle was significantly greater than docetaxel alone treatment in the xenograft models. Toxicity was significantly reduced in docetaxel nanoparticles conjugated with cetuximab compared to docetaxel nanoparticle alone.

The stability testing of prepared nanaoparticulate formulations was performed at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for six months and at long-term conditions (2-8°C)

up to six months. Various parameters, i.e. assay, entrapment, particle size and zeta potential, were evaluated after each predetermined time points (1, 2, 3 and 6 months).

The storage of the unconjugated and antibody conjugated drug nanoparticles of the drugs at 25°C ± 2°C/60% ± 5% RH, led to an increase in the particle size. The increase in the particle size was not significant during the first month, however became significant and more prominent after 2, 3 and 6 months. The polydispersity index of the nanoparticle stored at 25°C ± 2°C/60% ± 5% RH was found to increase as compared to the initial. The increase in the particle size may be due to the absorption of the moisture by the nanoparticles resulting in the coalescence of the small nanoparticles forming particles larger in size. The nanoparticles were also observed for physical appearance. After 3 and 6 months the physical appearance was also changed, with loss of the free flowing property followed by the difficulty in redispersibility. Water content was increased at accelerated condition while refrigerated condition maintained the water content value even after six months of storage. The drug content of the unconjugated and antibody conjugated drug nanoparticles was not changed at 6M at 5°C ± 3°C and, the drug content was reduced after 6M storage at 25°C ± 2°C/60% RH ± 5% RH. Hence, we can conclusively specify that both unconjugated and antibody conjugated nanoparticles of the three drugs were stable and can be stored 5°C ± 3°C for 6M retaining its original formulation characteristics.

8.2. Conclusions

To conclude, cetuximab conjugated docetaxel nanoparticles forms were successfully prepared.

The formulations were optimized to achieve maximum docetaxel encapsulation. Particle size of the nanoparticles was also chosen as one of the optimization parameters as particle size was important for the entry of nannoconstructs to tumor vasculature.

Presence the size of the nanoparticulate formulation was near 180 nm and the nanoparticles were round uniform in nature. The formulations showed less cell cytotoxicity at therapeutic and higher concentrations. Cetuximab conjugated docetaxel nanoparticles was found to increase the cell uptake of docetaxel. The nanoparticulate formulations prevent the toxic effect of the drug applied at high concentration of drugs and thus can increase the maximum tolerance dose (MTD). It is clear from the results that the docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles demonstrated higher cytotoxicity than the free drug formulation at the same drug concentration and exposure time, which means that for the same therapeutic effect, the drug needed for docetaxel nanoparticles and cetuximab conjugated Docetaxel nanoparticles formulation could be much less than that for the free drug.

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Present investigation shows a promising way to treat lung cancer using targeted drug delivery approach with enhanced margin of safety and reduced dose dependent toxicity of the docetaxel. It is suggested that approach will definitely open a vista in the era of cancer treatment with reduced dosing profile.