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Lung cancer is the third most common cause of death after heart disease and pneumonia globally. The prognosis in lung cancer patients is generally poor. About 80% of patients die within a year of diagnosis and only 5.5% are able to survive after five years. In 2006 alone in United States of America, lung cancer accounted for more deaths than combined deaths caused by breast cancer, prostate cancer and colon cancer. In the same year, 106,374 men and 90,080 women were diagnosed with lung cancer out of which, 89,243 and 69,356 women died due to lung cancer. There are two main types of lung cancer based on the characteristics of the disease and its response to treatment. Non-small-cell lung carcinoma (NSCLC) accounts for 80% of all lung cancers. NSCLC is divided into:
1. Squamous carcinoma is the most common type that accounts for 35% of all lung cancer cases. The cells are usually well differentiated and locally spread. Widespread metastases occur relatively late.  
2. Large-cell carcinoma accounts for 10% of all lung cancers. It is less well differentiated than the first type and metastasis earlier.  
3. Adenocarcinoma accounts for approximately 27% of lung cancers. It arises from mucous glands and from scar tissues. Metastases are common to the brain and bones. It is the most common type of lung cancer associated with asbestos and is proportionally more common in non-smokers, women and older people.  
4. Alveolar cell carcinoma, accounting for 1-2% of lung cancers.  
The second major type is the small-cell lung carcinoma (SCLC), which accounts for 20% of all lung cancers.  
Conventional approaches to treatment of lung cancer mainly comprise of surgery, radiation therapy and chemotherapy with cytotoxic agents. Surgery is a treatment option in some patients with stage I or II NSCLC in conjunction with radiotherapy and chemotherapy.  
A standard treatment method for patients with extensive-stage SCLC is combination chemotherapy, with or without prophylactic cranial irradiation (PCI). Extensive SCLC has been associated with an untreated median survival of only a few months. The use of combination chemotherapy, such as: 1-etoposide with cisplatin or carboplatin, 2-doxorubicin(DOX) and cyclophosphamide with etoposide or vincristine, and 3-cisplatin, Docetaxel, cyclophosphamide.
and etoposide are associated with a response rate of over 50% and a median survival of 10 months. The use of adjunctive radiation therapy does not help in extending survival in extensive disease. The drugs that are currently widely prescribed globally mainly include: Etoposide and Docetaxel alone and in combination with other cytotoxic drugs. Etoposide is an inhibitor of the enzyme topoisomerase II. It is used for malignancies such as lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme. It is often given in combination with other drugs such as cyclophosphamide and doxorubicin. However, the drug has got numerous side effects such as: low blood pressure, hair loss, metallic food taste, bone marrow suppression leading to leucopenia, anaemia, thrombocytopenia etc.

Docetaxel is a taxane derivative and is a semi-synthetic analogue of paclitaxel (Taxol®), an extract from the rare Western yew tree Taxus brevifolia. Due to scarcity of paclitaxel, extensive research was carried out leading to the formulation of docetaxel – an esterified product of 10-deacetyl baccatin III.

Docetaxel binds to microtubules reversibly with high affinity and has a maximum stoichiometry of 1 mole docetaxel per mole tubulin in microtubules. This binding stabilizes microtubules and prevents depolymerisation from calcium ions, decreased temperature and dilution, preferentially at the plus end of the microtubule.

The main use of docetaxel is in the treatment of a variety of cancers after the failure of anthracycline-based chemotherapy. Marketing of docetaxel as Taxotere® is mainly towards the treatment of breast, prostate and other non-small cell cancers. Clinical data has shown docetaxel to have cytotoxic activity against breast, colorectal, lung, ovarian, prostate, liver, renal and gastric cancer and melanoma cells.

Because docetaxel is a cell cycle specific agent, it is cytotoxic to all dividing cells in the body. This includes tumour cells as well as hair follicles, bone marrow and other germ cells. Haematological adverse effects associated with Docetaxel include neutropenia (95.5%), anaemia (90.4%), febrile neutropenia (11.0%) and thrombocytopenia (8.0%). Deaths due to toxicity accounted for 1.7% of the 2045 patients and incidence was increased (9.8%) in patients with elevated baseline liver function tests (liver dysfunction).
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Parenteral administration of chemotherapeutic agents results in non specific drug distribution and unleashes plethora of systemic side effects leading to poor quality of patient life. Since, last few decades, efforts have been concentrated in direction of using lung as a target organ for the treatment. There are salient advantages of direct, non invasive drug delivery to lungs since lungs offer a large surface area for absorption (~75m²); thin (0.1 to 0.5 μm) alveolar epithelium permitting rapid absorption, absence of first-pass metabolism, rapid onset of action, improved drug efficacy, reduced adverse drug reactions and high bioavailability.(Cryan, 2005) Site specific drug delivery to lungs serves as a major promise in targeting drugs in treatment of plethora of diseases viz. parasitic lung infections, (Chono, 2008) neoplastic pulmonary disorders (Hughes, 1989,Ahmad et al, 1993) and even genetic disorders like cystic fibrosis.(Alton, 1993)

In spite of availability of substantial number of natural, synthetic and semi-synthetic cytotoxic drugs for cancer chemotherapy, some of the major drawbacks associated with prevalent cancer chemotherapy are non specificity of anti cancer drugs precipitating out lethal toxicities at times leading to poor quality of life in surviving patients and failure to achieve optimal therapeutic concentrations in tumours. Hence, research has been focused on fabricating drug delivery systems that facilitate drug targeting to cancer affected tissues without undergoing non site specific distribution thereby minimizing the unwanted side effects and improving the prognosis.

Colloidal, vesicle based nanometric novel drug delivery systems such as nanoparticles; liposomes etc. can be fabricated to target the cytotoxic drug to the exact cancerous site. (Jain, 2008 Gupta, 2007). Strategies have been designed to take the advantage of the receptors and moieties that are largely over expressed in cancer cells and under expressed or nearly absent in normal, healthy tissues. Designing the colloidal vesicular drug delivery systems such as liposomes with sizes lesser than tumour size can be easily taken up and retained by tumours by virtue of Enhanced permeability and Retention (EPR) phenomenon and enhanced cellular uptake of cytotoxic drug via receptor mediated mechanisms. (Akima, 1996). Efforts are on to investigate and develop the ligands that can be directed towards such over expressed receptors and thereby achieve a highly target specific drug delivery. Attachment of ligand on liposomal surface will coax the liposomal delivery system only to tumours and not the adjacent healthy, non cancerous tissues.
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To lower overall cell toxicity and optimize therapeutic benefit to the cancer patients, targeted drug delivery systems for anti-tumour drugs have been developed. Drug delivery systems such as liposomes, microspheres, nanoparticles, immunoliposomes and lipoplexes are often directed against epitopes present on tumour cells and or receptors expressed on tumour cells and carry drugs interfering with tumour growth. Among these varied colloidal carriers, liposomes seem to be promising candidates. It has been demonstrated that small and stable liposomes can passively target several different tumours as they can circulate for longer times and extravasate in tissues with enhanced vascular permeability which is the case with tumours. Stealth and ligand conjugated liposomes serve as ideal candidates for tumour targeting as they can serve the purpose of achieving controlled and sustained drug delivery both.

Liposomes (PL bilayer vesicles) are the most advanced of the particulate drug carriers and are now considered to be a mainstream drug delivery technology. Both classical and stealth liposomes rely on "passive" targeting to increase the localization of anticancer drugs to solid tumours. Growing solid tumours, as well as regions of infection and inflammation, have capillaries with increased permeability as a result of the disease process (e.g., tumour angiogenesis). Pore diameters in these capillaries can range from 100 to 800 nm. Drug containing liposomes that have diameters in the range of approximately 60–150 nm are small enough to extravasate from the blood into the tumour interstitial space through these pores. Normal tissues contain capillaries with tight junctions that are impermeable to liposomes and other particles of this diameter. This differential accumulation of liposomal drugs in tumour tissues relative to normal cells is the basis for the increased tumour specificity for the liposomal drugs relative to free (non liposomal) drugs. In addition, tumours lack lymphatic drainage and therefore, there is low clearance of the extravasated liposomes from tumours. Passive targeting can result in manifold increase in drug concentrations in solid tumours relative to those obtained with free drugs. The mechanism of action of the liposomal drugs is thought to result from sustained release of drug from the liposomes and diffusion of the released drug throughout the tumour interstitial fluid, with subsequent uptake of the released drug by tumour cells.

In order to increase the specificity of interaction of liposomal drugs with target cells and to increase the amount of drug delivered to these cells, recent efforts in the liposome field have been focusing on the development of ligand-targeted liposomes (LTLs). These liposomes utilize
targeting moieties coupled to the liposome surface to selectively deliver the drug liposome package to the desired site of action. (Active targeting)

While ligands can be readily attached to the surface of either classical or stealth liposomes, ligand-targeted stealth liposomes have clear pharmacokinetic advantages over ligand targeted classical liposomes for in vivo applications, and the former are used almost exclusively for active targeting.

Certain tumors, including lung cancer, over express the CD44 cell-surface marker. CD44 is a receptor that binds to hyaluronan (HA), a carbohydrate consisting of β1,3 N-acetyl glucosaminyl-β1,4 glucuronide. We hypothesized that the incorporation of phosphatidylethanolamine lipid derivatives-containing HA oligosaccharides (HA-PE) into liposomes could target drug-containing liposomes to tumor cells that express CD44.

CD44 is found at low levels on epithelial, hemopoietic, and neuronal cells and at elevated levels in various carcinoma, melanoma, lymphoma, breast, colorectal, and lung tumor cells. This cell surface receptor binds to HA(hyaluronic acid) which is a high-M₆ glycosaminoglycan polymer (M₆, 1E6), composed of the repeating disaccharide β1,3 N-acetyl glucosaminyl-β1,4 glucuronide. HA is a major component of the extracellular matrix, CD44 is implicated in the metabolism of solubilized HA and is associated with metastatic dissemination of solid tumours. Although CD44 is expressed on a number of cell types in normal tissues, it turns out that these cell types are either not in direct contact with the blood or require activation before they bind to HA.

Strategies that interfere with CD44-HA interaction, such as the administration of high M₆ HA-anti-CD44 mAb, or a CD44-receptor globulin, reduce tumor formation in the lung for animal tumor models established from CD44-expressing tumor cell lines. Because the vascular system is leaky in many tumors so that HA-liposomes would gain access to the tumor cells subsequent to extravasating into the tumor from the circulation, CD44 may be a suitable surface receptor for targeted chemotherapy of cancers that express this receptor. High molecular weight HA-drug conjugates have been devised for this purpose.

HA is a potential ligand to target the tumor cells over expressing the CD44 receptor. Recently HA polymer drug conjugates have been used to deliver drugs to CD44 expressing cells.
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Certain tumors, including many that are found in the lung, over express CD44 cell-surface marker. CD44 is a receptor that binds to hyaluronan (HA) - carbohydrate consisting of β 1, 3 N-acetyl glucosaminyl-b1,4 glucuronide.

The huge strides in field of technology and science had largely led to development of unique routes of drug administration. Currently, pulmonary route is being largely explored for its potential role in improving drug delivery in lung disorders including malignancies. Treating respiratory diseases with inhalers requires delivering sufficient drug to the lungs to bring about a therapeutic response. For optimal efficacy, drug administration must be reliable, reproducible, and convenient. This goal can be achieved by a combination of formulation, metering, and inhaler design strategies.

The sustained drug delivery to the lung for local as well as systemic delivery is a new area of research based upon the engineering of particles which are inhaled to the lungs. The new field of therapeutic aerosol bioengineering driven primarily by the medical need for inhaled insulin, is now expanding to address medical need ranging from respiratory to systemic diseases, including asthma, growth deficiency, and pain. (Edwards et al., 2002) Bioengineering of therapeutic aerosols involves a level of aerosol particle design absent in traditional therapeutic aerosols, which are created by conventionally spraying a liquid solution or suspension of drug or milling and mixing a dry drug form into respirable particles. Aerosols have enabled several high-visibility clinical programs of inhaled insulin, as well as earlier-stage programs involving inhaled morphine, growth hormone, beta-interferon, alpha-1-antitrypsin, and several asthma drugs.

Currently, there exist three methods of targeted drug delivery to lungs:

1. Metered dose inhalers
2. Dry powder inhalers (DPI)
3. Nebulizers

DPIs offer a number of distinct advantages over traditional metered dose inhalers: DPIs are breath actuated; the energy for powder dispersion and generation of the aerosol is derived from patient’s inhalation. DPIs are free from problem of coordination of actuation and inhalation, are easy to formulate, propellant free and hence, ecofriendly.
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Demerits of DPIs mainly comprise of: poor dose uniformity, greater dependence on patient’s inspiratory flow and device dependence.

DPIs proved successful in addressing other device and formulation-related shortcomings of the pMDI. DPIs are easier to use, more stable and efficient systems. Because a pMDI is pressurized, it emits the dose at high velocity, resulting in increased incidence of premature deposition in the oropharynx. Thus, pMDIs require careful coordination of actuation and inhalation. Despite advancements in their design (e.g., use of spacers), incorrect use of pMDIs is still a prevalent problem.

Poor coordination of actuation and inhalation can cause decreased asthma control in a substantial proportion of patients. Since DPIs are activated by the patient’s inspiratory airflow, they require little or no coordination of actuation and inhalation. This has frequently resulted in better lung delivery than that achieved with comparable pMDIs. Since DPIs are typically formulated as one-phase, solid particle blends, they are also preferred from stability and processing standpoint. Dry powders are at a lower energy state, which reduce the rate of chemical degradation and the likelihood of reaction with contact surfaces. By contrast, pMDI formulations, which include propellant and co solvents, may extract organic compounds from the device components.

The large respirable particles present an opportunity to optimize pulmonary lung deposition, they disperse well from DPIs, and have been shown to improve peripheral (i.e., pulmonary or alveolar) lung deposition by reducing deposits in the extra thoracic (mouth and throat) and tracheo-bronchial airways and oropharyngeal passages rendering them ideal for inhaled therapies used in the treatment of “deep” lung diseases (e.g. asthma, cystic fibrosis), and systemic delivery (e.g., insulin). Liposomal DPIs have been indicated to exhibit reduced clearance by alveolar macrophage action, thereby improving the bioavailability of inhaled pharmaceuticals (Vanbever et al., 1999).

Here, in this work we have attempted to target the ligand-Hyaluronic acid (HA) grafted liposomes to facilitate site specific delivery of cytotoxic agent (Etoposide and Docetaxel) within lung cancer cells. Grafting of HA as a ligand to liposomal surface is believed to augment the affinity of grafted liposomes for lung cancer cells that over express CD44 receptors. HA grafted and non grafted drug loaded liposomes were studied for their efficacy and site specificity by performing cell cytotoxicity, cell uptake and cell cycle analysis in A549 cell lines. On the basis
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of superior performance exhibited by HA grafted drug loaded liposomes during cell line studies, the former were processed to DPIs. An attempt was made to develop liposome based aerodynamically light large porous particles which exhibit appreciable flowability, enhanced fine particle fraction, targeted and prolonged drug release, reduction in the extra thoracic and tracheobronchial deposition and avoidance of lungs natural clearance mechanisms. This facilitates deposition of liposomal DPIs containing cytotoxic drugs straightway to lung cancer cells and thereby enhance their site specificity, minimize drug induced adverse reactions and improve the overall survival rate, prognosis and quality of life of patients suffering from lung cancer.

1.1 RESEARCH ENVISAGED

The focus of the current investigation was to develop, characterize and optimize ligand grafted liposomes of cytotoxic drugs followed by assessing their efficacy and site specificity through cell line studies to get insight of ligand targeted drug loaded liposomes in targeting lung cancer. It was hypothesized to process optimized ligand grafted drug loaded liposomes to Dry Powder Inhalers (DPIs) and study in-depth the aspects related to their solid state characterization and in vitro lung deposition profile.

1.2 PROPOSED PLAN OF WORK

I. Literature survey pertinent to basic aspects of chemotherapy in lung cancer, liposomes in pulmonary drug delivery, dry powder inhalation formulation development technologies and profiles of selected drugs like Etoposide and Docetaxel.

II. Development and validation of analytical methods for estimation of drugs in solution, developed formulations, diffusion media, biological fluids and cell lysates.

III. Preparation of liposomes of drugs using thin film hydration method using lipids such as Hydrogenated Soya Phosphatidylcholine, Dipalmitoyl Phosphatidylethanolamine and cholesterol by applying suitable mathematical design.

IV. Optimization of liposomes in terms of percentage drug entrapment, particle size and zeta potential.

V. Preparation and optimization of ligand (Hyaluronic acid-HA) grafted drugs loaded liposomes.
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VI. Ex vivo evaluation of non grafted and HA grafted drugs loaded liposomes in A549 cell lines by assessing cell uptake, in vitro cytotoxicity, intracellular pharmacokinetic parameters and cell cycle pattern.

VII. Preparation and optimization of DPIs of optimized HA grafted drugs loaded liposomes.

VIII. Solid state characterization and in vitro lung deposition evaluation of developed liposomal DPIs of Etoposide and Docetaxel.

IX. Stability studies of optimized DPI formulations of HA grafted liposomally entrapped drugs in terms of physico chemical and biological (in vitro lung deposition) parameters.
1.3 REFERENCES

- Torchilin, V.P., 2006. Recent approaches to intracellular delivery of drugs and DNA and organelle targeting, Annual Review of Biomedical Engineering; 8: 343-375.
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