Chapter – 2

Review of Literature

Chapter 2 Literature Review

The lung is the body's organ of respiration. The trachea carries air into the body from the nose/mouth. It then splits into two bronchi which branch off into the left and right lungs. These primary bronchi branch into secondary bronchi, which in turn branch into tertiary bronchi which then become bronchioles. Each level of branching sees a decrease in diameter and an increase in the number of bronchioles. The bronchi have diameters > 5 mm while terminal bronchiole diameter can be as small as 5-10 μm. Their diameter is controlled by bronchial smooth muscle which is under autonomic control. At the end of the terminal bronchioles are the alveoli. The alveoli are the sites of gaseous exchange in the lung. Pulmonary capillaries surrounding the alveoli carry CO_2 and O_2 to and from the alveoli, respectively. The alveolar and micro capillary walls together make up what is known as the respiratory membrane. This consists of the alveolar epithelial lining (a layer of type I and type II alveolar cells and associated alveolar macrophages), an epithelial basement membrane, a capillary basement membrane and endothelial cells of the capillary. Despite having several layers, the respiratory membrane is very thin (~ 0.5 μm). This allows for the rapid diffusion of O_2 and CO_2 across the membrane. It has been estimated that the lungs contain over 3×10^8 alveoli giving it a surface area of about 70-100 m^2 for the exchange of gases.

In air-breathing animals, respiratory anatomy has evolved in such a way as to actively thwart inhalation of putative airborne particulates. The epithelium of the airways is a continuous sheet of cells lining the luminal surface. The airway epithelium has at least four major types of cells, including basal cells, ciliated cells, goblet cells and Clara cells. On the surface of the epithelium of the proximal respiratory tract, ciliated cells predominate. Together with basal cells and a small percentage of goblet cells, they form a pseudo stratified epithelium, in which ciliated cells occupy the majority of the luminal surface and basal cells are in contact with the basement membrane. Starting with the trachea and ending in the alveolar sacs, the thickness of respiratory epithelium decreases gradually from 60 μm in the tracheo bronchial epithelium to 0.2 μm in the alveolar region. Alveolar epithelial type I cells represent the principle cell type lining the surface of the alveoli. They are thin and broad representing 8.3% of the cell population within the

human lung. The major functions of these cells, which cover 93% of the alveolar space, are to provide a surface for gas exchange and to serve as a permeability barrier.

Alveolar epithelial type II cells have a much smaller surface area per cell and they represent 16% of the total cells in the lung. They play a basic role in synthesis, secretion and recycling of surface-active material (lung surfactant) and respond to alveolar injury by dividing, retaining morphological features of type II cells or differentiating into type I cells. Both type I and type II cells rest on the alveolar basement membrane that is nearly continuous. Epithelial cells are joined together by well-developed tight junctions. These junctions impart important permeability properties to the epithelial cell layer.

The alveolar region of the lung contains alveolar macrophages as well. These help in the clearance of inhaled particles. Alveolar macrophages are aid cells and serve well as primary defenders of the alveolar milieu.

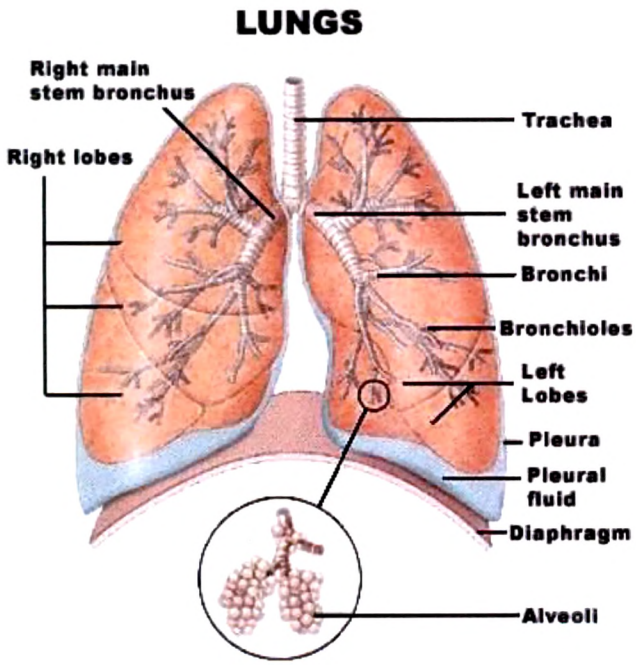

Fig. 2.1The anatomy of the human lung (taken from www.aduk.org.uk/gfx/lungs)

2.1 FACTORS AFFECTING PULMONARY DRUG DELIVERY

2.1.1 Mechanisms of particle deposition in airways

The adult human lung is exposed to more than 10,000 litres of air every day. Thus, with each intake of air, lung receives a high burden of dust, fumes, pollens, microbes and other contaminants. Efficient defence mechanisms have evolved to minimize burden of foreign particles entering the airways and clearing those that succeed in being deposited.

Devices employed for drug delivery to airways of lung generate an aerosol. Therapeutic aerosols are two phase colloidal systems in which drug is contained in a dispersed phase which may be a liquid, solid or combination of the two, depending upon the formulation and method of aerosol generation. Drug must be presented to the lung in aerosol droplets or particles that deposit in the appropriate lung regions and in optimum quanta to be effective.

Once the aerosol particle or droplet has deposited in the lung, there are a number of barriers which must be overcome before the drug exerts its pharmacological effect. The respiratory defence mechanism of mucociliary clearance and phagocytosis by macrophages may act upon undissolved particles. Aerosol particle dissolution may be slow and drug may then be subsequently subjected to enzymatic degradation before it reaches its site of pharmacological action.

Three principal deposition mechanisms operating within the lower respiratory tract:

a. Inertial compaction

This is the dominant deposition mechanism for particles >1 μm in the upper tracheo bronchial regions. A particle with a large momentum may be unable to follow the changing direction of the inspired air as it passes the bifurcation and consequently, collide with the airway walls as it continues on its original course. Impaction generally occurs near bifurcations, probability of inertial impaction will be dependent upon particle momentum, thus particles with larger diameters or higher densities and those travelling in

airstreams of higher velocity will show greater impaction. Airflow velocities in main bronchi are estimated to be 100-fold higher than in the terminal bronchioles.

b. Sedimentation

This is particle deposition resulting from settling under gravity. It becomes increasingly important for particles that reach airways where the airstream velocity is relatively low. e.g. the bronchioles and alveolar region. The fraction of particles depositing by this mechanism will depend upon the time the particles spend in these regions.

c. Brownian diffusion

Particles below 1 μm usually get displaced by a random bombardment of gas molecules, resulting in particle collision with the airway walls. The probability of particle deposition by diffusion increases as the particle size decreases. Brownian diffusion is also more prevalent in regions where airflow is less or absent.

As a consequence of these physical forces acting on the aerosol particle, its deposition in lungs is highly dependent on diameter. It has been observed that:

Particles with size more than 10 μm will impact in the upper airways and are rapidly removed by coughing, swallowing and mucociliary clearance.

Smaller particles in size range of 0.5-5 μm may escape impaction in upper airways and will deposit by impaction and sedimentation in lower tracheo bronchial regions.

2.1.2 Physiological factors affecting particle deposition in airways

2.1.2.1 Lung Morphology

Each successive generation of tracheo bronchial tree produces airways of decreasing diameter and length. Each bifurcation results in an increased probability for impaction and decrease in airway diameter is associated with a smaller displacement required for a particle to contact a surface. Thus to travel down the airways, drug particles must pass through successive series of branching tubes of constantly decreasing size. The aerosol

particles must constantly change direction in order to remain airborne. Thus lobes of the lung which have the shortest average pathlength will show greatest peripheral deposition.

Inspiratory flow rate

Increasing the Inspiratory flow rate (IFR) will enhance deposition by impaction in the first few generations of the tracheo bronchial region. Increase in flow will not only increase particle momentum but will also result in an increase in turbulence, particularly in the pharynx and trachea, which itself will enhance impaction in the proximal tracheo bronchial region. Air velocity also depends upon physical activity and estimated airflow velocities resulting from sedentary activities. The effects of IFR on deposition from devices which use the energy of inspiration in order to generate the drug aerosol (most DPIs) are more complex, since an increase in IFR will in most cases lead to the production of an aerosol of smaller particle size.

Co ordination of aerosol generation with inspiration

The momentum of aerosol particle generated from pressurized metered dose inhalers is largely governed by pMDI formulation rather than subject's IFR. pMDI aerosol droplets will be travelling at velocities of 2500-3,000 cm/sec. A failure to co ordinate actuation of pMDI during the early phase of the inspiratory maneuver will result in near total particle impaction in the oropharyngeal region. Deposition from pMDIs may be increased at higher IFR as a result of better aerosol entrainment and lower deposition in the throat.

Tidal Volume

An increased IFR will usually be associated with an increase in volume of air inhaled in one breath, the tidal volume. Clearly, an increase in tidal volume will result in penetration of aerosol particles deeper into the tracheo bronchial region and a greater chance for deposition within these regions.

Breath Holding

Increasing the time between the end of inspiration and start of exhalation increases the time for sedimentation to occur. Breath holding is commonly used to optimize pulmonary drug delivery. For maximum effect, breath holding for a period of 5-10 seconds post inspiration is recommended. Under inhaled conditions, a 5 μm particle will settle a few mm during a 5-second breath hold.

Diseased states

Bronchial obstruction as seen in a variety of pulmonary disorders will be associated with greater local airflows and turbulence and this will result in localized deposition in the large airways of the trachea bronchial region. Bronchoconstriction of asthma has a great influence on exhalation than inhalation and thus deposition by sedimentation may be greater than normal.

2.1.3 Pharmaceutical factors affecting aerosol deposition

Aerosol velocity

The aerosols produced by nebulizers and dry powder inhalers (DPIs) are transported to lungs by entrainment on the inspired air and thus their velocity are determined by the inspiratory maneuver and lung physiology. In contrast, pMDIs generate aerosol droplets with velocities greater than inspiratory airflow and consequently the aerosol will have a greater tendency to impact in the oropharyngeal region.

Particle size

Commercial devices do not lead to monodispersed particles and often the size distribution is wide and particles may exhibit varying shapes. Therefore, a number of terms are used to adequately characterize an aerosol sample:
Particle size is conventionally defined as the aerodynamic diameter, which is the diameter of a spherical particle with unit density that settles at the same rate as the particle in question.

The mass median aerodynamic diameter (MMAD) is defined as the aerodynamic diameter which divides the aerosol mass distribution in half. The geometric standard deviation (GSD) is defined as the size ration at 84.2% on the cumulative frequency curve to the median diameter. This assumes that the distribution of particles is log normal. A monodisperse aerosol has a GSD of 1, although in practice, aerosols with a GSD>1.22 are referred to as polydispersed or heterodispersed. The MMAD and GSD of aerosols are hence, critical in determining the deposition patterns within the lungs. Aerosols with larger MMADs will deposit higher in the respiratory tract since the aerosol particles will have greater momenta. A polydispersed aerosol is also likely to show greater deposition in tracheo bronchial than a monodisperse aerosols of the same MMAD.

Shape

Particles which are non spherical will have atleast one physical dimension which is greater than the aerodynamic diameter.

Density

Particles with density lesser than 1 g/cm^3 will have a mean physical diameter greater than the aerodynamic parameter. Most micronized drugs for inhalation will have particle densities around 1, although materials produced by lyophilization or spray drying are likely to be significantly less dense. Large porous particles with physical diameters of 20 μm and densities of 0.4 g/cm^3 are efficiently deposited in lungs.

Physical stability

Therapeutic aerosols are often inherently physically unstable since they have a high concentration of particles and their close proximity may lead to mutual repulsion or other inter particulate reactions. Aerosol particles generated by DPIs may be hygroscopic and, during their passage through the high humidity environments of airways, may increase in size and thus have a greater chance of being prematurely deposited.

2.2 FATE OF PARTICLES IN THE AIRWAYS

2.2.1 Mucus barrier

The first barrier which is encountered before the drug can reach its site of action is the mucus, present as viscoelastic layer in the tracheo bronchial region. If the drug is given as an aerosolized powder then the drug first needs to dissolve in the mucus layer. Although mucus has very high water content, varying between approximately 90-95%, its viscosity may result in a slow dissolution of drugs. Thus dissolution may be a rate determining step, especially for poorly soluble drugs and steroids which are delivered as dry powder aerosols. Improvement of drug penetration into mucus has been attempted using mucolytics drugs such as N-acetylcysteine, which act to reduce mucus viscosity. Highly water soluble drugs, given as dry powder inhalers, may dissolve at the very high relative humidity (>99%) present in airways air and impact as solution droplets.

Once in solution, drug will diffuse through the mucus layer and enter the aqueous environment of the epithelial lining fluid. The rate of diffusion through the mucus will be dependent upon factors such as:

Thickness of mucus layer.

Mucus viscosity

Molecular size of drug

Drug-mucosal interactions including the binding of positively charged drug molecules to mucus glycoproteins via electrostatic interactions with the negatively charged silaic acid residues as well as hydrogen bonding and hydrophobic interactions.

2.2.2 Mucociliary clearance

In the healthy lung, mucus layer does not exist as a stagnant layer but is constantly being propelled along the tracheo bronchial airways by rhythmic beating of cilia on epithelial cells to the central bronchi, trachea and then to the throat where the mucus and any entrapped particles are swallowed. In the tracheo bronchial region a higher proportion of

the epithelial cells are ciliated such that there is a near completer covering of the central airways by cilia. Towards the periphery of the tracheo bronchial region cilia are less abundant.

Mucociliary clearance is an organized, complex process which is highly dependent upon the composition and depth of the epithelial lining fluid and the viscoelastic properties of the mucus. In many airways disease there is hyper secretion of mucus. This may cause an overloading of the ciliary transport process resulting in a debilitated mucociliary clearance and the build up of mucus as a thick, highly viscous layer.

2.2.3 Alveolar clearance

In the alveolar region, deposited particles may be engulfed by alveolar macrophages and cleared then from the lung by a number of different routes. The main route is via the mucociliary escalator, although transport from the alveolar region to the start of the mucociliary escalator is a very slow process and may involve transport by random movement through macrophages. Macrophages may also be transported via lymphatic systems to lymph nodes and bloodstream.

2.3 FACTORS AFFECTING THE ABSORPTION AND METABOLISM OF DRUGS IN THE AIRWAYS

It is important to consider the absorption and metabolism of drugs administered to lungs whether they are given to elicit a local or systemic response. Absorption is clearly important for systemically acting drugs since it is one element of the events leading to delivery of drugs to its site of action. Absorption is equally important for locally acting drugs since for these compounds it represents removal of drug from its site of action. Metabolism of drugs is also an important consideration since it may lead to drug inactivation or production of active or toxic metabolites.

2.3.1 Area

The architecture of lung is designed for highly efficient exchange of gases. These same features also offer great potential for the delivery of systemically acting compounds. The

surface area of airways is approximately 140 m^2, slightly larger than that of the small intestine. A well designed aerosol system can rapidly deliver drug to a high proportion of surface area, whereas an orally delivered drug will have its access to the small intestine delayed by gastric emptying.

2.3.2 Absorption barrier thickness

Thickness of absorption barrier is much smaller than for most other routes of drug delivery. In some parts of the alveolar region the airways to blood pathlength is less than 0.5 μm, an order of magnitude thinner than typical mucosal or epithelial membranes. This property facilitates very rapid transfer of gases, vapours and other small molecules. Drug absorption is usually more rapid than form any other epithelial routes of delivery. The absorption barrier thickness in the tracheo bronchial region of the lung is much thicker than in the alveolar region but the absorption of drugs from this region is still likely to be much quicker than from any other mucosal route of delivery.

2.3.3 Blood supply

The lung receives 100% of the cardiac output via a network of fine capillaries. This rich blood supply which promotes rapid gaseous exchange is also beneficial for systemic drug delivery. Drugs absorbed from the lung pass directly to the heart avoiding first pass metabolism in the liver, although some drugs will be subjected to first pass metabolism during absorption in lungs.

2.3.4 Membrane permeability

The main permeability barrier in the airways lies with the epithelium of lung airway rather than instertitium or endothelial lining of the capillaries. The epithelial permeability towards hydrophilic solutes is atleast 10-fold lower than that of the endothelium. The epithelium of the lung is much more permeable than that of the other mucosal routes. For example, less than 3% of an oral dose of sodium chromoglycate reaches the circulation whereas more than 70% is absorbed from lung into bloodstream after inhalation.

In the alveolar region the tight junction gap between type-I alveolar cells is reported as 1 nm. Other pores with equivalent radii of about 10 nm have also been identified. Consequently, the permeability of the paracellular route is much greater than seen with other membranes. Large molecules upto 150 kDa are reported to be absorbed to a small extent into the bloodstream after pulmonary administration.

2.3.5 Enzymatic activity

The endothelial cells of the lung play an important role in the metabolism of certain endogenous compounds and most of the drug metabolizing enzymes found in liver, also occur in lungs. Many isozymes of the cytochrome P-450 family have been identified in the respiratory tract with the highest concentrations of these occurring in the nasal mucosa and smaller airways with lower levels in trachea and bronchi. In the lung these isozymes are most concentrated in Clara cells, type-II cells and to a lesser extent in macrophages.

Non cytochrome P-450 dependent enzyme systems, including esterases, peptidases, flavin containing monooxygenases are also present in the lungs. Their distribution tends to be more widespread and their activities much higher than is seen with P-450 systems.

Locally acting inhaled drugs may be inactivated by these enzymes, for example isoprenaline and rimiterol are metabolized by catechol-o-methyl transferase. The inhaled steroid beclomethasome propionate is hydrolyzed by esterases, firstly to an active metabolite, beclomethasone monopropionate, and then to an inactive metabolite, beclomethasone.

Inhaled drugs intended for systemic action are likely to be subjected to some first pass metabolism during their absorption from the lung. The extent of this pre systemic first pass metabolism in the lung has not been fully quantified for many drugs but is estimated to be far less than that seen in the gastro intestinal tract and liver after oral dosing.

2.4 MERITS AND DEMERITS OF PULMONARY DRUG DELIVERY

A brief overview of both the merits and demerits of pulmonary drug delivery is given below.

2.4.1 Locally acting drugs

Delivery of anti asthmatic and other locally acting drugs directly to the site of action in lungs is associated with following advantages:

- Reduction in dose needed to produce a pharmacological effect.
- Low concentration in the systemic circulation associated with reduced systemic side effects
- Rapid onset of action.
- Avoidance of gastro intestinal upset.
- Avoidance of intestinal and hepatic first pass metabolism.
- Local administration is also associated with certain demerits for these drugs:
- Oropharyngeal deposition may give local side effects.
- Patients may have difficulty using the delivery devices correctly.

2.4.2 Merits and demerits for systemically acting drugs

For the delivery of systemically acting drugs not suitable for delivery via oral route, lungs offer a number of potential advantages:

- Lungs offer a very large surface area for drug absorption.
- Permeability of lung membranes towards many compounds is higher than that of the small intestine and other mucosal routes.
- Highly vascular surface of the alveolar region promotes rapid absorption and onset of action.

- Lung offers a much less hostile atmosphere than the oral route to most drugs, including proteins and peptides.

Demerits of lungs for delivery of systemically acting drugs include:

- Lungs are not readily accessible surfaces for drug delivery. Complex delivery devices are required to target drugs to the airways and these devices may be inefficient.
- Aerosol devices can be difficult to use; for example, it has been estimated that approximately 50% or more adults have difficulty using conventional pMDIs efficiently even after careful training. Dexterity is also required which may be lacking in very young and elderly patients.
- Various factors affect the reproducibility of drug delivery to the lungs including physiological (respiratory maneuver) and pharmaceutical variables.
- Drug absorption may be limited by the physical barrier of the mucus layer and the interactions of drugs with mucus.
- Mucociliary clearance reduces the retention time of drugs within lungs. Efficient drug delivery of slowly absorbed drugs must overcome the ability of the lung to remove drug particles by mucociliary transport.

2.5 LUNG CANCER

The thorax is a common site of involvement by primary and metastatic malignancy. The lung is unique among all organs in having a very high degree of exposure to the internal environment through the pulmonary blood flow and from the outside by exposure to air flow. Patients suffering from metastatic cancer have circulating cancer cells in their blood that are shed from the primary tumour. Hence, the lungs are continually exposed to these neoplastic cells. Fortunately, the natural defence mechanism in the lungs scavenges most, if not all, of the cells that are deposited in the pulmonary capillaries and interstitium. However, a few cells survive and proliferate into metastases. Thus, the lungs are a common site of metastatic disease.

Chapter 2 Literature Review

Lung cancer, which includes cancer of the trachea and bronchi, is the third most common cause of death in the UK after heart disease and pneumonia. It is responsible for around a quarter of all cancer deaths. Pamela Mason reported that the mortality rate worldwide is highest in Scotland, closely followed by England and Wales. In England and Wales, lung cancer was responsible for nearly 29,000 deaths in 2002 with a male to female ratio of approximately two to one. The lung cancer incidence and death figures in UK are worse than other European and USA averages.

Although the mortality rate for this disease has levelled off in men, it is still the most common cause of cancer death in this population group, and men account for 60% of all lung cancer cases. The incidence continues to arise in women accounting for one in six of all cancer deaths. This is directly related to changes in smoking habits. In women, lung cancer is generally the second most common cause of cancer death after breast cancer. Risk increases with age - lung cancer is less common in people under the age of 40. Trends in lung cancer incidence and mortality reflect smoking habits and/or exposure to other environmental or occupational carcinogens. The incidence rate in men is 34.9 per 100,000 with highest rates observed in more developed countries although mortality rate has gone down in countries in which male tobacco consumption has declined. In women, incidence rates are lower (11.1 per 100.000) with the highest rates found in North America and Northeastern Europe, but there is a rising trend in incidence and mortality. The prognosis in lung cancer patients is generally poor. About 80% of patients die within a year of diagnosis and only 5.5% are alive after five years. This is due to the speed with which the disease progresses and also to the nature of the patients, most of whom are older and often suffer from smoking related illnesses, including chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Overall, patients with metastatic disease to the lung as well as those with locally advanced primary lung cancers carry poor prognoses and are not usually amenable to curative therapy with surgery or with chemotherapy or radiation.

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 accounting 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 metastasizes earlier.

3. Adenocarcinoma, which 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. Arising from endocrine cells, these tumours secrete many polypeptide hormones. Some of these hormones provide feedback to the cancer cells and cause tumour growth. This type of tumour grows rapidly, taking approximately three years from initial malignant change to presentation.

Patients with lung cancer often do not exhibit specific symptoms, particularly in early stage disease. Dyspnoea, cough and thoracic pain are early signs, while haemoptysis often indicates advanced disease. Relapsing infectious diseases of the respiratory system in combination with a smoking history suggest a need for further diagnostic investigations, including medical history and physical examination, laboratory tests, chest radiography, thoracic CT or MRI, bronchoscopy and biopsy. For staging according to UICC criteria, additional CT or MRI of the abdomen and the brain, bone scan, and eventually positron emission tomography are used. Serum tumor marker measurements also potentially have an important role in both diagnosis and staging.

For patients with NSCLC, particularly those with early stage disease (Stages I to IIIA), surgery is the mainstay of treatment. The additional application of adjuvant radio- or chemotherapy after tumor resection has previously been shown to have only limited benefit. However, more recent data indicate a considerable improvement in overall survival when modern adjuvant chemotherapies are applied. The use of neoadjuvant systemic therapies prior to surgery to provoke tumor shrinkage and early eradication of systemic micro metastases is still under discussion. Five-year-survival rates depend strongly on tumor stage; with 60%-70% five-year survival reported for patients with Stage I disease, 40%-50% for Stage II and 15-30% for Stage IIIA. Median survival for Stage IV disease patients has been stable for years at 8 to 10 months. Although response rates for chemo- and radiotherapy are low, several studies have demonstrated moderate beneficial effects concerning survival, time to disease progression and quality of life as compared with best supportive care.

Small cell lung cancer is characterized by its rapid doubling time and propensity for early metastases. In clinical practice only two stages of SCLC are distinguished: limited stage disease with tumor confined to one hemi thorax only and extensive stage disease with metastases in the contra lateral chest or at distant sites. Approximately 20 to 25% of patients have limited disease, treatable with curative intent. However, 5-year survival rates are still low (15-25%, compared with <5% in extensive disease). In these patients, multimodal approaches of chemo and radiotherapy are recommended followed by prophylactic cranial irradiation to prevent cerebral metastases.

The intensive search for new therapeutic drugs in advanced lung cancer is highlighted in the 2003 ASCO guidelines for the treatment of NSCLC, which for patients with good performance score suggest second and third-line therapies that were not available when previous recommendations were made in 1997.

With the prospect of more effective therapeutic options for advanced stage disease, current follow-up procedures for lung cancer should perhaps be reviewed. Tumor marker

measurements, which potentially provide sensitive and cost-effective early detection of recurrence, may become increasingly important in assessing the efficacy of therapy.

Surgery is a treatment option in some patients with stage I or II NSCLC. Radiotherapy and chemotherapy can be offered. Due to the importance of the lung for survival, it is often not possible to remove the tumours completely without dramatically reducing lung function. Thus, lung tumours are treated by radiation therapy or chemotherapy. Both types of treatment cause painful toxicity to the patient that may require a premature end of the therapy even though the tumour cells are successfully killed. The reason for this is that all cytotoxic drugs kill normal cells as well as cancerous cells unleashing severe and at times, fatal side effects. Furthermore, because of the blood circulation in the body, only a small fraction of the drug actually reaches the target tumour and most of the drug acts on normal tissues or is rapidly eliminated. Therefore, to obtain a therapeutic effect, a relatively high dose of drug must be administered and usual drug formulations are used in a balance between killing the tumour (efficacy) and killing the patient (toxicity).

Chemotherapy plays an important role in treating many patients with both NSCLC and SCLC. For patients with early-stage NSCLC, drugs can be used either following surgery (i.e. adjuvant chemotherapy) or before surgery is carried out (i.e. neoadjuvant chemotherapy). The goal of chemotherapy is to help "cure" the patient and improve long term survival rates. Patients with SCLC and advanced NSCLC can also benefit from chemotherapy, with the aim of drug treatment being to prolong life, improve or maintain quality of life and control symptoms without causing unacceptable toxicity.

One way to reduce the toxicity of the drug to the normal tissue is to direct the drug to the tumour cells with a drug delivery system. This is similar in concept to the use of a cruise missile to destroy only a military target while leaving surrounding buildings intact. Targeted drug delivery to lung tumours may prove to be the most efficacious and economical means by which to treat lung cancer, since small groups of tumour cells that have spread away from the main tumour and are too few in number to be detected, can be exposed to a high level of drug by these "smart bombs". The bomb in this research is

called a liposome. The liposome is a microscopic balloon, smaller than a red blood cell, formed from lipids. Cytotoxic drugs can be carried inside the liposome, where they do not come in contact with the cells. The lipids can be broken down by metabolism inside the body and the drug is then released. The technology for encapsulating drugs in liposomes has been thoroughly worked out by many laboratories around the world.

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, DOX, cyclophosphamide and etoposide, is associated with a response rate of over 50% and a median survival of 8-12 months. The use of adjunctive radiation therapy does not help in extending survival in extensive disease.

Optimal timing, dose and fractionation of radiotherapy treatment have yet to be defined. For extensive SCLC, the treatment of choice is combination chemotherapy, usually cis- or carboplatin and etoposide. Current approaches include drugs such as topoisomerase I inhibitors and taxanes.

2.6 DRUG PROFILE

2.6.1 Etoposide

Etoposide is a semi synthetic derivative of podophyllotoxin that exhibits antitumor activity. Etoposide inhibits DNA synthesis by forming a complex with topoisomerase II and DNA. This complex induces breaks in double stranded DNA and prevents repair by topoisomerase II binding. Accumulated breaks in DNA prevent entry into the mitotic phase of cell division, and lead to cell death. Etoposide acts primarily in the G2 and S phases of the cell cycle.

Synonyms: (-)-Etoposide, Etoposidum [INN-Latin], trans-Etoposide

Brand Names: Eposin, Etopophos, Lastet, Toposar, Vepesid J, Zuyeyidal

2.6.1.1 Physico Chemical Aspects

Physical properties: Solid, white amorphous powder, sensitive to light.

Melting point: 236-251^{0}C.

Water Solubility: 58.7 mg/L

logP : 1.16

pKa : 12.28

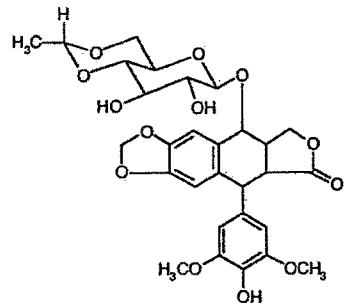

Fig. 2.2 Chemical Structure of Etoposide

2.6.1.2 Pharmacological Aspects

Indication

Etoposide is indicated with other chemotherapeutic agents in the treatment of refractory testicular tumors and as first line treatment in patients with small cell lung cancer, malignancies such as lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme.

Etoposide is an antineoplastic agent and an epipodophyllotoxin (a semisynthetic derivative of the podophyllotoxins). It inhibits DNA topoisomerase II, thereby ultimately inhibiting DNA synthesis. Etoposide is cell cycle dependent and phase specific affecting

mainly the S and G2 phases. Two different dose-dependent responses are seen. At high concentrations (10 μg/mL or more), lysis of cells entering mitosis is observed. At low concentrations (0.3 to 10 μg/mL), cells are inhibited from entering prophase. It does not interfere with microtubular assembly. The predominant macromolecular effect of etoposide appears to be the induction of DNA strand breaks by an interaction with DNA-topoisomerase II or the formation of free radicals.

Mechanism of action

Etoposide inhibits DNA topoisomerase II, thereby inhibiting DNA re-ligation. This causes critical errors in DNA synthesis at the premitotic stage of cell division and can lead to apoptosis of the cancer cell. Etoposide is cell cycle dependent and phase specific, affecting mainly the S and G2 phases of cell division.

Pharmacokinetics

Absorption on oral administration

When orally administered, drug absorption is largely dose dependent. Absorption decreases as dose increases.

Daily doses exceeding 220 mg should be divided prior to administration.

Absorption is not affected by food, stomach pH.

The drug shows overall good absorption.

Time to peak plasma concentration (Tmax): is 1-1.5 hrs.

Mean bioavailability: 50%.

Distribution

Etoposide undergoes widespread tissue distribution. The drug is detected in saliva, liver, spleen, kidneys and myometrium.

Volume of Distribution (Vd) : 7-17 L/m^2 (32 % of body weight of a normal, healthy adult)

Plasma Protein Binding: Highly protein bound (97%)

Half Life: 4-12 hours

Metabolism

Drug primarily undergoes hepatic metabolism at large (through O-demethylation via the CYP450 3A4 isoenzyme pathway) with 40% excreted unchanged in the urine.

Cytochrome P450 3A 4 is responsible for 3'-demethylation reaction converting Etoposide to 3'-demethyl etoposide.

Route of Elimination

Etoposide is cleared by both renal and non renal processes, i.e., metabolism and biliary excretion. Glucuronide and/or sulfate conjugates of etoposide are also excreted in human urine. Biliary excretion of unchanged drug and/or metabolites is an important route of etoposide elimination as fecal recovery of radioactivity is 44% of the intravenous dose. Only 8% or less of an intravenous dose is excreted in the urine as radiolabeled metabolites of 14C-etoposide.

Excretion of Etoposide also follows fecal elimination. Drug is largely (40-60%) excreted out via urine, 16% by fecal route and 6 % by billiary route.

Clearance: 33 – 48 ml/min [IV administration]

Adverse Drug Reactions

Low blood pressure, hair loss, metallic food taste, bone marrow suppression leading to leucopenia, anaemia, thrombocytopenia etc. are normally seen in patients treated with Etoposide.

2.6.1.3 Dose and Route of Administration

Intravenous (IV) Dose: 35 mg/m^2/day for 4 days to 50 mg/m^2/day for 5 days.

Commercially available dosage forms with respective route of administration

Oral: Capsule

IV: Liquid and solution

2.6.1.4 Marketed Preparations

VePesid 20 50 mg capsule

Etopophos 100 mg vial

Etoposide 50 mg capsule

Etoposide 100 mg/5 ml vial

Toposar 100 mg/5 ml vial

Toposar 1000 mg/50 ml vial

Toposar 500 mg/25 ml vial

2.6.2 Docetaxel

Docetaxel is of the chemotherapy drug class; taxane, 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, which is extracted from the renewable and readily available.

Docetaxel differs from paclitaxel at two positions in its chemical structure. It has a hydroxyl functional group on carbon 10, whereas paclitaxel has an acetate ester and a tert-butyl substitution exists on the phenylpropionate side chain. The carbon 10 functional group change causes docetaxel to be more lipid soluble. 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 than paclitaxel.

Docetaxel is a clinically well established anti-mitotic chemotherapy medication used mainly for the treatment of breast, ovarian and non-small cell lung cancer. Docetaxel binds to microtubules reversibly with high affinity and has a maximum stoichiometry of 1 mole docetaxel per mole tubulin in microtubules.

2.6.2.1 Physico Chemical Aspects

Physical state: White, fluffy solid powder.

Melting point: 232^0C

Solubility: practically insoluble in water (0.025 mg/L)

logP :2.4

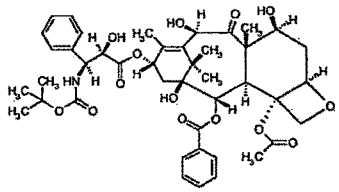

Fig. 2.3 Chemical Structure of Docetaxel

2.6.2.2 Pharmacology

Indication

Docetaxel is mainly prescribed for the treatment of patients with locally advanced or metastatic breast cancer after failure of prior chemotherapy. Also used as a single agent in the treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of prior platinum-based chemotherapy and lastly, for use in combination with prednisone and in the treatment of patients with androgen independent (hormone refractory) metastatic prostate cancer.

Pharmacodynamics

Docetaxel is a taxoid anti neoplastic agent. It promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. In addition, docetaxel induces abnormal arrays or "bundles" of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis.

Mechanism of action

Docetaxel interferes with the normal function of microtubule growth. Whereas drugs like colchicine causes the depolymerization of microtubules in vivo, docetaxel arrests their function by having the opposite effect; it hyper-stabilizes their structure. This destroys the cell's ability to use its cytoskeleton in a flexible manner. Specifically, docetaxel binds to the β-subunit of tubulin. Tubulin is the "building block" of mictotubules, and the binding of docetaxel locks these building blocks in place. The resulting microtubule/docetaxel complex does not have the ability to disassemble. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a transportation highway for the cell. Chromosomes, for example, rely upon this property of microtubules during mitosis. Further research has indicated that docetaxel induces programmed cell death (apoptosis) in cancer cells by binding to an apoptosis stopping protein called Bcl-2 (B-cell leukemia 2) and thus arresting its function.

Pharmacokinetics

Volume of distribution (Vd): 113 L

Protein Binding: About 94% protein bound, mainly to α-acid glycoprotein, albumin and lipoproteins.

Clearance

21 L/h/m^2 [Cancer patients after IV administration of 20–115 mg/m^2]

Metabolism

Drug undergoes extensive hepatic metabolism. In vitro drug interaction studies revealed that docetaxel is metabolized by the CYP3A4 isoenzyme (1 major, 3 minor metabolites).

Route of elimination

Docetaxel is eliminated in both the urine and feces following oxidative metabolism of the tert-butyl ester group, but fecal excretion was the main elimination route. Within 7 days, urinary and fecal excretion accounted for approximately 6% and 75% of the administered radioactivity, respectively.

Toxicity or Adverse Drug reactions

Oral LD_{50} in rat is >2000 mg/kg. Anticipated complications of over dosage include: bone marrow suppression, peripheral neurotoxicity, and mucositis. In two reports of overdose, one patient received 150 mg/m^2 and the other received 200 mg/m^2 as 1-hour infusions. Both patients experienced severe neutropenia, mild asthenia, cutaneous reactions, and mild paresthesia, and recovered without incident.

Incidence of commonly experienced non-haematological adverse effects reported for treatment with docetaxel. Haematological adverse effects 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).

2.6.2.3 Dose and Route of Administration

Docetaxel is available in market as Intravenous solution only. (Mainly marketed as Taxotere®)-20 mg/0.5 ml.

2.7 LIPOSOMES

Liposomes were discovered in the early 1960s by Bangham and colleagues (Bangham et al, 1965). However, it took several years from the early to the late 60s before the system was realized as a potential drug carrier. At first they were used to study in vivo simulated biomembrane behavior. Subsequent to that liposome has become an essential therapeutic tool most notably in drug delivery and targeting. Liposomes have covered predominantly medical and drug delivery areas like tumor targeting, gene and antisense therapy, genetic vaccination, Immunomodulation, lung therapeutics, fungal infections and skin care and topical cosmetic products. (Gregoriadis, 1971, Szoka and Papahadjoplous, 1978)

Structurally, liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids.

Mechanism of liposome formation

Phospholipids are amphipathic (having affinity for both aqueous and polar moieties) molecules as they have a hydrophobic tail and a hydrophilic or a polar head. The hydrophobic tail is composed of two fatty acid chains containing 10-24 carbon atoms and 0-6 double bonds in each chain. The polar end of the molecule is mainly phosphoric acid bound to a water soluble molecule. The hydrophilic and hydrophobic domains/segments within the molecular geometry of amphiphilic lipids orient and self organize in ordered supramolecular structure when confronted with solvents.

The most common natural polar phospholipid is phosphatidylcholine (PC). Formation of bilayered structures facilitates reduction in free energy and thereby renders the bilayered vesicles (liposomes) thermodynamically stable. At lower water content and higher temperature, varieties of lyotropic liquid crystalline phases also exist. Exposure of lipids to temperature equal to or slightly above phase transition results in transformation of structural integrity of lipids from a tightly packed, rigid form to a relatively loosely bound, flexible and significantly disordered physical form. At this stage, phospholipids

bilayers are capable of enclosing the drug or therapeutic entities within their interior. Cholesterol is an important constituent in liposomes. Cholesterol plays a crucial role in maintaining the bilayered structure of liposomes (Papahadjoupoulos et al, 1973; Kirby and Gregoriadis, 1980 and New 1989).Cholesterol, by virtue of its amphiphillic nature and relatively smaller size, orients itself within the space adjacent to phospholipids. However, cholesterol itself is not capable of initiating bilayered structure; it is quintessential for maintaining structural integrity and rigidity of the liposomes. By the same mechanism, it also prevents the tilting and distortion of membrane structure at temperatures exceeding phase transition temperatures. Liposomes are often classified on the basis of the vesicular size and the number of lamellae present. Based on the number of lamellae, they can be classified as unilamellar and multilamellar vesicles.

Depending on the nature of the polar head group, phoshoplipid liposomes may be either charged or uncharged. For charged liposomes, colloidal stability is determined largely by the magnitude and range of electrostatic interactions, also for zwitterionic phospolipids with a zero net charge, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), phospholipid liposomes repel each other, thereby providing colloidal stability to such systems. Liposomes have been applied to the field of drug delivery in numerous diverse directions.

2.7.1 COMPOSITION OF LIPOSOMES

2.7.1.1 Phospholipids

Glycerol containing phospholipids are by far, the most commonly used component of liposome formulations and represent more than 50% of the weight of lipid present in biological membranes. Natural lipids such as egg lecithin, soya lecithin, and synthetic lipids such as phosphoglycerolipids, sphingolipids, and digalactosylglycerolipids can be mentioned as examples of potentially useful lipids. Amongst natural lipids may be mentioned sphingolipids such as sphingomyelin, ceramide and cerebroside; galactosylglycerolipids such as digalactosyldiacylglycerol; phosphoglycerolipids such as egg-yolk phosphatidylcholin and soya-bean phosphatidylcholin ; and lecithins such as

egg-yolk lecithin and soya-bean lecithin Amongst synthetic lipids may be mentioned dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine, dilauryl phosphatidylcholine, 1-myristoyl-2-palmitoyl phosphatidylcholine, 1-palmitoyl-2- myristoyl phosphatidylcholine, dioleoyl phosphatidycholine, hydrogenated soyaphosphotidylcholines (HSPC), and the like. Some naturally occurring phospholipids include phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylglycerol (PG) while dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylserine (DPPS), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidicacid (DPPA), dipalmitoyl phosphatidylglycerol (DPPG), dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidylglycerol (DOPG) are some synthetic phospholipids.

2.7.1.2 Sterols

Sterols such as cholesterol, ergosterol, nanosterol, or its derivatives are often included as components of liposomal membrane. Cholesterol has been called the "mortar" of bilayer because by virtue of its molecular shape and solubility properties, it fills in empty spaces among the phospholipid molecules, anchoring them more strongly into the structure. Its inclusion in liposomal membranes has 3 effects (i) increasing the fluidity or microviscosity of the bilayer (ii) reducing the permeability of the membrane to water-soluble molecules and (iii) solubilizing the membrane in the presence of biological fluids such as plasma.

2.7.1.3 Other Non-Structural Components

Charge inducer materials which provides a negative charge, for example phosphatidic acid, dicetyl phosphate or beef brain ganglioside etc, or one which provides a positive charge for example stearylamine acetate or cetylpyridinium chloride etc. have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Many single chain surfactants of number of single and double chain lipids having fluorocarbon chains and also compounds like quaternary ammonium salts and dialkyl phosphates can also be used to form liposomes.

2.7.2 Types of Liposomes

Different types of liposomes can be prepared and are classified by the size and structure. Different types of liposomes are small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), oligolamellar vesicles (OLV), and multi-lamellar vesicles (MLVs). MLVs consist of numerous concentric bilayers separated by aqueous spaces and range up to 15 μm in diameter. Vesicles consisting of a single bilayer encompassing a central aqueous compartment are referred to as small unilamellar vesicles (SUVs), which range upto 100 nm in diameter and large unilamellar vesicles (LUVs) ranging from 100 to 500 nm in diameter.

2.7.3 Methods of Preparation of Liposomes

Numerous procedures have been developed to prepare liposomes. There are at least fourteen major published methods for making liposomes (Ostro et al, 1989; Martin et al, 1990). The seven, most commonly employed methods are: lipid film hydration method (Bangham et al, 1965), Ethanol injection method (Batzri et al, 1973), Ether infusion method (Deamer et al. 1976), Detergent dialysis method (Kagawa et al, 1971), French press method (Barenholz et al, 1976), Rehydration-dehydration techniques (Shaw et al, 1985) Reverse phase evaporation method (Szoka et al, 1978).

Liposomes can be prepared by number of processed in which water soluble or hydrophilic materials are entrapped by using aqueous solution of these materials as hydrating fluid or by the addition of liposomes. The oil soluble, lipophilic materials are solubilized in the organic solution of the constitutive lipids and then evaporated to dry drug containing lipid film followed by its hydration. These methods involve the loading of the entrapped agents before or during the manufacturing procedure (passive loading). However, certain types of compounds with ionizable groups and those which display both lipid and water solubility can be introduced into the liposomes after the formation of intact vesicles (remote loading).

2.7.3.1 Passive loading techniques

Passive loading techniques include three different groups of methods working on different principles viz. mechanical dispersion, solvent dispersion and detergent solubilization.

Mechanical dispersion methods of passive loading

These methods involve preparation of a lipid solution in an organic solvent that finally ends up in dispersing the lipids in water. The various components are combined by co - dissolving lipids in an organic solvent and the organic solvent is removed by film deposition under vacuum. When all the solvent is removed, the solid lipid mixture is hydrated using aqueous buffer. The lipids spontaneously swell and hydrate to form liposomes. At this juncture, methods incorporate some diverse processing parameters in various ways to modify their ultimate properties. These post hydration treatments include vortexing, sonication, freeze thawing and high pressure homogenization.

2.7.3.1.1 Thin film hydration using hand shaking (MLVs) and non shaking methods (ULVs)

In these methods, lipids are casted as stacks of film from their organic solution using rotary flash evaporator under reduced pressure (or by hand shaking) and then the casted film is dispersed in an aqueous medium. Upon hydration, the lipids swell and peel off from the wall of the round bottom flask and vesiculate forming multilamellar vesicles (MLVs). The mechanical energy required for swelling of lipids and dispersion of casted lipid film is imparted by manual agitation or by exposing film to a stream of water saturated nitrogen for 15 minutes followed by swelling in swelling in aqueous medium without shaking (non shaken vesciles).The percentage encapsulation efficiency is as high as 30%. However, large amounts of water soluble compounds are wasted during swelling as only 10-15% of the total volume gets entrapped. On the other hand, lipid soluble compounds can be encapsulated at 100% efficiency, provided they are present in adequate quantities and do not disturb structural composition of the membrane.

Sonicated Unilamellar Vesicles (SUVs)

At high energy level, average size of vesicles is further reduced. This was first achieved on exposure of MLVs to ultrasonic irradiation and still remains the method of choice for preparing small size vesicles. There are two methods of sonication based on the use of either probe or bath ultrasonic disintegrators. The probe is usually employed for dispersions requiring high energy in small volume while the bath is generally suitable for larger volumes of diluted lipids. Probe tip sonicators supply a high energy input to the lipid dispersion but suffer from overheating of the liposomal dispersion cause lipid degradation. Sonication tips also tend to release titanium particles into the liposome dispersion which must be removed by centrifugation prior to use. Because of these apparent reasons, bath sonicators are most widely used for the preparation of SUVs. Sonication of MLV dispersion is accomplished by placing a test tube containing the dispersion in a bath sonicator and sonicating for 5-10 minutes (10,000 g) above the Tc of the constituent lipid. The lipid dispersion should begin to clarify to yield a slightly hazy transparent solution. The haze is due to light scattering induced by residual large particles remaining in the dispersion. These particles can be removed by centrifugation to yield a clear SUV dispersion.

Liposomal dispersion after centrifugation is placed in a clear plastic walled centrifuge tube. The dispersion is generally centrifuged at 10,000g (30 minutes, 20^0C) to sediment titanium particles and large MLVs followed by higher speed centrifugation (1, 59,000g, for 3-4 h). After spinning the tube, it is carefully removed from the rotor and with the help of a Pasteur pipette, liquid with top clear layer is decanted leaving the central opalescent layer (containing small multilamellar vesicles) and a pellet behind. The top layer constitutes pure dispersion of SUVs with varying diameters in nanometric range as the size and distribution is affected by composition and concentration, temperature, sonication time and power, volume and sonication tuning.

French Pressure Cell Liposomes

The ultrasonic radiation not only degrades the lipids but also macromolecules and other sensitive compounds that are to be entrapped in liposomes. One of the earliest and still useful methods developed is extrusion of preformed liposomes in a French press under very high pressure. This technique yields uni or oligo lamellar liposomes of intermediate size range. These liposomes are more stable as compared to sonicated liposomes. The method however, suffers from drawbacks of being costly and variability in size of liposomal product.

The liposomes prepared by this technique are less likely to suffer from structural defects and instabilities as observed in sonicated vesicles. Leakage of contents from liposomes prepare using French press is slower and lower than sonicated liposomes. French press has also been used to reduce the heterogeneity of population of proteoliposomes obtained by detergent dialysis technique.

Microemulsification Liposomes (MEL)

Microfluidizer is used to prepare small MLVs from concentrated lipid dispersion. Microfluidizer pumps the fluid at very high pressure (10,000 psi, 600-700 bar) through a 5 μm orifice. Then it is forced along defined micro channels, which directs two streams of fluid to collide together at right angles at a very high velocity, thereby affecting an efficient transfer of energy. The lipids can be introduced into the fluidizer, either as a dispersion of large MLVs, or as slurry of non hydrated lipid in an organic medium. The fluid collected can be recycled through the pump and interaction chamber until vesicles of the spherical dimension are obtained.

After a single pass, size of vesicles is reduced to a size of 0.1 and 0.2 μm in diameter. The exact size distribution, however, depends on the nature of the components of the membrane and hydration medium. The presence of negative lipids tends to decrease their size, while increasing cholesterol concentration gives larger liposomes. In addition to the high rate of production, this method has the advantage of being able to process samples

with a very high proportion of lipids (20% or more by weight). This process is efficient for encapsulation of water soluble materials.

Freeze Thaw Sonication (FTS) method

This method is based on freezing of unilamellar (mainly SUV) dispersion and then thawing by standing at room temperature for 15 minutes and finally subjecting to a brief sonication cycle. Thus the process ruptures SUVs during which solute equilibrates between inside and outside, and the liposomes themselves fuse and increase in size.

Solvent Dispersion Methods for Passive loading

In solvent dispersion method, lipids are first dissolved in an organic solution, which is then brought in contact with an aqueous phase containing materials to be entrapped within liposomes. The lipids align themselves at the interface of organic and aqueous phase forming monolayer of phospholipids, which forms the half of the bilayer of the liposome. Methods employing solvent dispersion can be categorized on basis of the miscibility of the organic solvent and aqueous solution. These include the conditions where the organic solvent is miscible with the aqueous layer; the organic solvent is immiscible with the aqueous phase, the latter being in excess; and the cases where the organic solvent is in excess, and immiscible with aqueous phase.

Ethanol Injection

This method has been reported as one of the alternatives used for the preparation of SUVs without sonication. An ethanol solution of lipids is injected rapidly through a fine needle into an excess of saline or other aqueous medium. The rate of injection is sufficient to achieve complete mixing, so that the ethanol is diluted almost instantaneously in water and phospholipid molecules are dispersed evenly throughout the medium. This procedure yields a high proportion of SUVs, although lipid aggregates and larger vesicles may form if the mixing is not thorough enough. This method is extremely simple and has low risk of degradation of lipids. The vesicles of 100 nm size may be

obtained by little modification in this method, i.e. by varying the concentration of lipid in ethanol or by changing the rate of injection of ethanol in preheated aqueous medium.

The major shortcoming of the method is the limited solubility of lipids in ethanol and volume of ethanol that can be introduced into medium, which in turn limits quantity of lipid dispersed, so that the resulting liposomal dispersion gets diluted. Another drawback is difficulty in removal of residual ethanol from phospholipid membrane.

Ether Injection

It is similar to the ethanol injection method; however, it contrasts markedly with ethanol injection in many respects. It involves injecting the immiscible organic solution very slowly into an aqueous phase through a narrow needle at the temperature of vapourizing the organic solvent. This method may also treat sensitive lipids very gently. It has little risk of causing oxidative degradation provided ether is free from peroxides. The disadvantages of the technique are the long time taken to produce a batch of liposomes and a careful control needed for introduction of the lipid solution, requiring a mechanically operated pump. If substances are degraded at elevated temperature, then the fluorinated hydrocarbons may be used instead of ether.

Rapid Solvent Exchange Vesicles (RSEV)

This is a recently introduced method adopted for preparation of liposomes. In this method, lipid mixture is quickly transferred between an essentially pure solvent environment and a pure aqueous environment. This method is specifically designed to form compositionally homogeneous dispersion by sudden precipitation of lipid mixture in an aqueous buffer. Phospholipid/cholesterol dispersion turns to be free of artifactual crystals when prepared by rapid solvent exchange method.

The method involves passing the organic solution of the lipids through the orifice of blue-tipped syringe (injection needle) under the vacuum into a tube containing aqueous buffer. The tube is mounted on the vortexer. Bulk solvent vaporizes and is removed within seconds before coming in contact with aqueous environment, while the lipid mixture

rapidly precipitates in aqueous buffer. Since the method is devised specifically for the fast and efficient removal of organic solvent, it does not require a highly volatile solvent. RES liposomes require not more than a minute for preparation and manifest high entrapment volumes with a high fraction of external surface with no evidence of artifactual demixing as observed with other conventional dispersion methods.

De-emulsification methods

This method requires two steps for preparation of liposomes, first the inner leaflet of the bilayer, then the outer half. The common feature of this method is the formation of "water in oil" emulsion by introducing a small quantity of aqueous medium containing material to be entrapped into a large volume of immiscible organic solution of lipid. This was followed by mechanical agitation to break up the aqueous phase into microscopic water droplets. These droplets are stabilized by the presence of phospholipid monolayer at the interface. The size of droplets is determined by the intensity of mechanical energy used to form the emulsion and amount of lipid relative to the volume of aqueous phase, since each droplet requires a complete monolayer of phospholipid covering its surface in order to prevent the possible coalescence with other droplets. The aqueous solution surrounded by monolayer of phsopholipid forms the central core of the final liposome. There are number of methods which could be used for preparing droplets including double emulsion, reverse phase evaporation and sonication methods.

Reverse Phase Evaporation vesicles

The salient feature of this method is the removal of a solvent from an emulsion by evaporation. The droplets are formed by bath sonication of mixture of two phases, and then the emulsion is dried down to a semisolid gel in a rotary evaporator under reduced pressure. At this stage, the monolayers of phospholipids surrounding each water compartment are closely opposed by each other and in some cases probably already form part of a bilayer membrane separating adjacent compartments. The next step is to bring about the collapse of a certain proportion of water droplets by vigorous mechanical shaking using a vortex mixer. In these circumstances, the lipid monolayer, which

enclosed the collapsed vesicle, is contributing to adjacent intact vesicle to form the outer leaflet of the bilayer of a large, unilamellar liposome. The aqueous content of the collapsed droplet provides the medium required for dispersion of these newly formed liposomes. After conversion of the gel into a homogeneous free flowing fluid, the dispersion is dialyzed to remove the last traces of solvent. The vesicles formed are unilamellar and have an average diameter of 0.5 μm. The encapsulation percentage is found to be nearly 50%.

Stable Plurilamellar Vesicles (SPLVs)

This method involves preparation of water in organic phase dispersion with an excess of lipid followed by drying under continued bath sonication with an intermittent stream of nitrogen. The redistribution and equilibration of aqueous solvent and solute occurs in between the various bilayers in each plurilamellar vesicle. The internal structure of SPLVs is different from that of MLV-REVs, in that they lack a large aqueous core, the majority of the entrapped aqueous medium being located in the compartment in between adjacent lamellae.

Detergent Depletion (Removal) methods of passive loading

In this method, phospholipids are brought in intimate contact with the aqueous phase via detergents, which associate with phospholipids molecules and serve to screen the hydrophobic portions of the molecule from water. The structures formed as a result of this association are known as micelles, and can be composed of several hundreds of component molecules. Their shape and size depend upon the chemical nature of the detergent, concentration and other lipids involved. The concentration of detergent in water at which micelles just start to form is known as the Critical Micelle Concentration (CMC). Below the CMC, detergent molecules exist entirely in free solution. As detergent is dissolved in water in concentration higher than the CMC, micelles form in more and more numbers, while the concentration of detergent in the free form remains essentially the same as it is at the CMC. Micelles containing other participating components in addition to the detergent in their formation are called mixed micelles.

Dialysis

In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media and there is equilibrium between the detergent molecules in aqueous phase, and in the lipid environment of the micelle. The critical micelle concentration can indicate the position of the equilibrium. Upon lowering the concentration of detergent in bulk aqueous phase, molecules of detergent can be removed from mixed micelles by dialysis. A higher CMC indicates that the equilibrium is strongly shifted towards the bulk solution, so that the removal form the mixed membrane by dialysis becomes relatively easy.

Detergents commonly used for this purpose exhibit reasonably high CMC (10-20 mM) so that their removal is facilitated. They include the bile salts sodium cholate and sodium deoxycholate and synthetic detergents such as octylglucoside.

Column Chromatography

Phospholipids in the form of either sonicated vesicles or as a dry film, at a molar ratio of 2:1 with deoxycholate form unilamellar vesicles of 100 nm on removal of deoxycholate by column chromatography. This could be achieved by passing the dispersion over a Sephadex G-25 column pre saturated with constitutive lipids and pre equilibriated with hydrating buffer.

Detergent Adsorption using Bio-Beads

Detergent (non ionic)/phospholipid mixtures can form large unilamellar vesicles upon removal of non ionic detergents (Triton X-100) using appropriate adsorbents for the detergent.

2.7.4 Remote (Active) Loading

Utility of liposomes as drug delivery systems is stimulated with the advancement of efficient encapsulation procedures. The membrane from lipid bilayer is in general impermeable to ions and larger hydrophilic molecules. Ions transport can be regulated by the ionophores while permeation of neutral and weakly hydrophobic molecules can be

controlled by concentration gradients. Some weak acids or bases however, can be transported through the membrane due to various transmembrane gradients such as electrical, ionic (pH) or specific salt (chemical potential) gradients. Several methods exist for improved of the drugs, including remote (active) loading of the drugs into preformed liposomes using pH gradients and potential difference across liposomal membranes. A concentration difference in proton concentration across the membrane of liposomes can drive the loading of amphipathic molecules. Active loading methods have the following advantages over passive encapsulation techniques:

- A high encapsulation efficiency and capacity.
- A reduced leakage of the encapsulated compounds.
- "Bed side" loading of drugs those limiting loss of retention of drugs by diffusion, or chemical degradation during storage.
- Flexibility for the use of constitutive lipids, as drug is loaded after the formation of carrier units.
- Avoidance of biological activity compounds during preparation steps in the dispersion thus reducing safety hazards.

Transmembrane pH gradient can be developed using various methods depending upon the nature of the drug to be encapsulated.

Weak amphipathic bases accumulate in the aqueous phase of lipid vesicles in response to a difference in pH between inside and outside of the liposomes. The pH gradient is created by preparing liposomes with a low pH inside and outside the vesicles, followed by addition of the base to extraliposomal medium. Usually, a two step process generates this pH imbalance and remote loading; first vesicles are prepared in a low pH solution, thus generating a low pH within the liposomal interiors followed by addition of the base to extraliposomal medium. Basic compounds, carrying amino groups are relatively lipophilic at high pH and hydrophilic at low pH. In a two chambered aqueous system

separated by a membrane, accumulation occurs at the low pH side, under dynamic equilibrium conditions. Thus the unprotonated form of basic drug can diffuse through the bilayer. At the low pH side the molecules are predominantly protonated, which lowers the concentration of drug in unprotonated form, and thus promote the diffusion of more drug molecules at the low pH side of the bilayer. The second step involves the exchange of external medium by gel exclusion chromatography with a neutral solution. Weak bases like doxorubicin, adriamycin and vin cristine which co exist in aqueous milieu in neutral and charged forms have been successfully loaded into preformed liposomes via the pH gradient method.

2.8 CHARACTERIZATION OF LIPOSOMES

Liposomal formulation after their formulation and processing for a specified purpose are characterized to ensure their predictable in vitro and in vivo performances. The liposomes produced by different techniques may have different physico chemical characteristics. The characterization parameters for the purpose of evaluation could be classified into three broad categories, which include physical, chemical and biological parameters. Physical characterization evaluates various parameters including size, shape, surface features, lamellarity and phase behavior and drug release profile. Chemical characterization includes those studies which establish the purity and potency of various liposomal constituents. Biological characterization parameters are helpful in establishing the safety and suitability of the formulations for the in vivo use for therapeutic applications.

Some of the parameters characterized in liposome product development are size and size distribution, surface topology, encapsulation efficiency, capture volume, lamellarity and in vitro drug release profile.

2.8.1 Vesicle shape and lamellarity

Vesicle shape can be assessed using various electron microscopic techniques which can be extended to determine the average size of vesicles. The lamellarity of MLVs is

heterogeneous and usually it is unilamellar as well as multilamellar. Earlier lamellarity calculations were based on techniques that detected proportion of lipids exposed to the external medium. This led to approximately half of the total lipid in LUVs and an even smaller fraction in MLVs. Labelling or binding studies are now employed to determine the proportion of outer monolayer lipid. However, lamellarity of the vesicles, i.e. the number of bilayers present in the liposomes is determined using Freeze fracture electron microscopy and ^{31}P nuclear magnetic resonance analysis.

Freeze fracture and freeze etch electron microscopy

Freeze fracture electron microscopy can be used not only to assess shape and lamellarity but also the surface morphology of the liposomes. In this technique the fracture plan passes through the vesicles, which are randomly positioned in the frozen state. Thus, the fracture plan may not necessarily pass through the mid plane and thus non mid plane fracture may result in erroneous readings. Freeze fractured specimens can provide information about fractures of vesicles that are unilamellar in a given population. After 5 minutes of etching, cross fractured vesicles are clearly seen and number of lamellae can readily be determined.

2.8.2 Vesicle size and size distribution

The average vesicle size and size distribution are important parameters as far as in vitro characteristics are concerned. Various techniques are described in literature for determination of size and size distribution. These include light microscopy, electron microscopy and freeze fracture microscopy, field flow fractionation, gel permeation and gel exclusion and zeta sizer.

2.8.2.1 Microscopic Techniques

Optical Microscopy

This technique mainly comprises of Brightfield, phase contrast microscopy and fluorescent microscopy and are useful in evaluating the vesicle size of large vesicles (>1 μm) particularly the upper end of the size distribution for multilamellar vesicles.

Negative stain Transmission Electron Microscopy (TEM)

This technique can be used to assess liposomal shape and size. Scanning electron microscopy (SEM) requires dehydration of sample prior to examination and is less preferred. Negative stain electron microscopy visualizes relatively electron transparent liposomes as bright areas against dark background. Liposomes are embedded in this method in a thin film of electron dense heavy metal stain.

Scanning Electron Microscopy (SEM)

It is less frequently used, although SEM has been reported in the literature. The distortion during sample preparation can be cited as the reason of non acceptability of SEM as one of the evaluation method. Scanning tunneling microscopy or atomic force microscopy on the other hand offer excellent improvisation in resolution. Monolayers under water can be resolved at the level of individual molecules using these techniques.

Diffraction and Scattering Techniques

Laser Light Scattering

Laser based quasi elastic light scattering techniques are useful in analyzing the homogeneous colloidal particles. The technique is based on the time dependent coherence of light scattered by a vesicle sensitive to diffusion, which in turn is dependent upon the viscosity of the aqueous medium and vesicle size. This technique can be applied to unimodel systems with mean diameter less than 1 μm.

Photon correlation spectroscopy is the major technique based on laser light scattering analysis that exploits the time dependence of intensity fluctuations in scattered laser light due to the different Brownian motions of the particles in liposomal dispersion. This differential diffusion profile of the particles of small and larger dimensions accordingly influences the rate of fluctuations of scattered light intensity, which is a function of mean hydrodynamic radius of the particles determined using Stokes-Einstein equation.

2.8.3 Surface Charge

Liposomes are generally prepared using charge imparting constitutive lipids and hence it is imperative to study the charge on vesicle surface. In general two methods are used to assess the charge, viz. free flow electrophoresis and zeta potential measurement. From the mobility of the liposomal dispersion in a suitable buffer (determined using Helmhotz-Smoluchowski equation), surface charge on vesicle can be computed.

2.8.4 Encapsulation Efficiency

The term depicts the percent aqueous phase and hence the percentage of water soluble drug that gets ultimately entrapped during preparation of liposomes and is usually expressed as percentage entrapment/mg lipid. Encapsulation efficiency is assessed using two techniques including minicolumn centrifugation and protamine aggregation method.

Minicolumn centrifugation is generally used both as a means of purification and separation of liposomes on a small scale and analysis of a liposomal dispersion to determine encapsulation efficiency. A Sephadex or Sepharose column pre saturated with the dispersion medium in 1.0 ml disposable syringe is run while applying liposomal dispersion (200 µl) first and saline (250 µl) thereafter and centrifuging the column at 2000 rpm for 3 minutes and assaying the elutes. Depending upon the different molecular weights of entrapped solids, various media and their cut off point should be chosen. The concentration of free or entrapped material in elutes can be determined by disrupting liposomes using ethanol (2 ml ethanol for 10 µl of liposomes) or Triton X-100 (10 µl of 10% Triton X-100 for 10 µl of liposomes) and estimating the liberated contents using standard methods.

Protamine aggregation method may be used for neutral and negatively charged liposomes. Liposomal dispersion (100 µl) can be precipitated with a protamine solution (100 µl, 10 mg/ml) and subsequent centrifugation at 2000 rpm. By analyzing the material in the supernatant and in the liposomal pellet (after disrupting liposomal pellet with 0.6

ml of Triton X-100), the encapsulation efficiency of the entrapped material can be estimated.

2.8.5 Trapped Volume

Trapped volume is an important parameter that governs the encapsulation efficiency and morphology of the vesicles. The best way to measure internal volume is to measure the quantity of water directly and this may be done by replacing the external medium with a spectroscopically inert fluid (Deuterium Oxide, D_2O) and then measuring the water signal, for example using NMR. The permeability of liposomal membrane to water is such that H_2O and D_2O equilibrate rapidly throughout the whole volume of the medium. The NMR scan of this medium can be used to assess the peak height, which can be related to concentration by comparison with standards containing known amount of H_2O in D_2O. A novel method based on calculation of intra vesicular volumes by salt entrapment has also been explored to determine trapped volume.

2.8.6 Phase Response and Transitional Behavior

Liposomes and lipid bilayers exhibit various phase transitions that are studied for their roles in triggered drug release or stimulus mediated fusion of liposomal constituents with target cells. Lipid bilayers can exist in a low temperature solid ordered phase and above a certain temperature in a fluid disordered phase, the temperature of this phase transition can be tailored by selecting proper lipids. Various phase transition and fluidity of phospholipid membranes is important both in manufacture and exploitation of liposomes, since the phase behavior of a liposomal membrane determines properties such as permeability, fusion, aggregation and protein binding. Moreover, these phase behaviours are important to characterize while formulating the liposomes with lipids having different phase transition temperatures and for polymer (PEG) grafted liposomes where polymer grafting as such provides a stealthing effect deterring from macrophagic uptake and hence, prolonged circulation.

2.8.7 Stability of liposomes

Stability of any pharmaceutical product is the capability of the delivery system in the prescribed formulation to remain within defined or pre established limits for a pre determined period of time (shelf life of the product). There is no established protocol for either accelerated or long term stability studies for the liposomal formulations though many of them are available in the market. However, the traditional guidelines that are generally observed for pharmaceutical dosage forms are followed while adopting the environmental conditions of the institutional setup. The stability aspects could be broadly studied in two main sections: First, the in vitro stability, which covers the stability aspects prior to administration of the formulation and with regard to the stability of the constitutive lipids. Second, the in vivo stability which covers the stability aspects once the formulation is administered via various routes to biological fluids. These include stability aspects in blood administered by systemic route or in gastro intestinal route, if administered by oral or peroral routes.

Stability

Hydrated bilayered vesicles are not considered to be thermodynamically stable and are thought to represent a metastable state in that the vesicles possess an excess of energy. Liposomal phospholipids can undergo chemical degradation such as oxidation and hydrolysis. Liposomes maintained in aqueous dispersion may aggregate and as a result may dump their contents.

Apart from that, method of formulation, nature of amphiphile and encapsulated drug molecules manipulate membrane rigidity and permeability characteristics. Leakage of hydrophilic drugs from aqueous domains of the liposomal bilayers upon storage is a common problem and needs to be addressed during in vitro stability studies.

In vitro stability mainly covers the chemical stability of the constitutive lipids under the various accelerated or long term storage conditions. Chemically, phospholipids are susceptible to hydrolysis. Additionally, phospholipids containing unsaturated fatty acids

are vulnerable to oxidative degradation and peroxidation. Categorizing stability as physical or chemical is not sufficient; unless a detailed shelf life stability protocol is designed and conducted.

2.9 DRUG DELIVERY TO TUMOUR

Cancer therapeutics is largely confined to directly encountering the tumour cells. The current cytotoxic agents mainly interfere with processes involved in cell growth while the aim of immunotherapy is to make immune effector cells to selectively attack the tumour cells. A recent approach to fight tumours is to impede and interfere with its blood supply, i.e. turning off angiogenesis or neovascularization.

To lower cytotoxicity and increase therapeutic efficacy, targeted drug delivery systems for anti tumour drugs have been developed for last few years. These delivery systems mainly comprise of: liposomes, nanoparticles, microspheres etc. often directed against epitopes present on tumour cells and/or receptors expressed on tumour cells and carry drugs that interfere with tumour growth. The bio active has to cross the tumour blood vessel wall consisting of endothelial cells and basement membrane. The focus of research changes from manipulation in barrier function of carrier system or designing of carrier systems that can cross tumour vasculature, to identification of recognition elements on tumour cells that could be targeted using suitable ligands anchored on these systems. The vasculature endothelium, basement membrane and tumour stroma may contain potential tumour specific targets. Strategies directed to these potential targets are aimed at interfering with blood vessel permeability, angiogenesis or tumour blood supply, or at manipulating endothelial cell mediated immune effector cell movement into the tumour tissue.

2.9.1 Development of tumour

Tumour seems to arise from the effects of different kinds of carcinogens. One of these categories comprises of agents that damage genes involved in controlling cell proliferation and migration. Cell adhesion is a pre requisite for cell survival in the normal

condition and is thought to occur via anchoring of cells within themselves or with extracellular matrix and allow cells to survive and proliferate. Tumour cells however, survive without adhesion to extracellular matrix. Tumours arise when a single cell accumulates a number of mutations, usually over many years and finally form most restraints on proliferation. The mutations allow the cell and its descendants to develop additional alterations and to accumulate in increasingly large numbers, forming a tumour that consists of most of these abnormally grown cells. Another category of agents does not damage the genes but instead selectively enhance the growth of tumour cells or their precursors. The primary danger of malignancies is that they can metastasize, allowing some of the cells to migrate and thus carry the disease to other parts of the body. Epithelial tumours or carcinomas are most common malignancies. The tumour mass emerges as a result of mutations in genes and genetically altered cells.

2.9.2 Molecular Targets for Tumour Therapy

Molecular targets on surface membrane of malignant cells may conveniently be divided into following categories and could be targeted using the counter ligand or specially designed antibodies. (Vyas, 2001)

- Altered expression of cell adhesion and their ligands.
- Altered expression of certain receptors otherwise expressed by all eukaryotic cells, like insulin receptors and MHC class-I associated compound receptors.
- Exquisite expression of receptors during certain stages of cellular differentiation, like transferrin receptor (TfR), folate receptors, apo-lipoprotein receptor, c-kit receptors, haemopexin receptors and MHC class-II associated compound receptors.
- Altered expression of certain growth factors (epidermal growth factor receptor, EGFr) and certain vasoactive and angiogenic peptides.
- Expression of tumour vasculature epitopes, either of the endothelial cells or of the basement membrane supporting the endothelial cells or tumour stroma components.

- Expression of surface determinants on malignant cells like Ig antigens and tumour associated antigens (TAA).

Targeting at the cellular level is highly desirable in tumour chemotherapy where therapeutic indices are insignificant with conventional therapy. Success seems to rely on some tumour cell associated receptors that are either expressed at extremely low density level or not expressed at all on the tumour cells.

There is a search of new glycoprotein and glycolipid tumour markers and/or tumour specific carbohydrate antigens. Heterogeneity in tumour population raises a note of caution in the quest of cellular targeting regimen based on highly specific tumour markers. Tumour cells epitopes exploited in cellular targeting are mainly based upon either tumour cell surface receptors or TAA and glycol conjugate markers. Expression of malignancy stigmata (i.e. tumourogenicity and metastatic activity) of the proliferating cell lines is a function of the interaction between cell membrane located receptors and epitopes in cellular microenvironment. Tumour progression may lead to activation and expression of a number of normal genes, including those for growth factors and ligand specific receptors, which are otherwise not expressed on normal cell lineage. The molecular receptors on the cell surface membrane may be divided into three categories:

Receptors expressed during certain stages of cellular differentiation like transferrin receptor (TfR), folate receptors, epidermal growth factor receptors (EGFR), apolipoprotein receptor, c-kit receptors and many more.

The receptors can either be over expressed or down regulated in the malignant target sites. Depending upon the over expression (folate, transferrin, fucose and lipoprotein) or down regulation (asialoglycoprotein receptor, mannose receptor), effective cellular targeting approaches could be engineered.

Epitopes on Tumour Vascular Endothelium for selective drug delivery

Tumour endothelial cells are suitable targets for targeted drug delivery and immunotherapy as they are accessible through the blood. The epitopes present on the

tumour endothelial vasculature is the form of angiogenic peptides and adhesion molecules that interplay between cells, soluble factors and extracellular matrix component could be exploited to negotiate targeting.

2.9.2.1 Targeted drug delivery via folate receptor

The folate receptor is a highly selective tumour marker over expressed in more than 90% of ovarian carcinomas and epithelial tumours and has been used as a targeting ligand. Two general strategies have been developed for the targeted delivery of drugs to folate receptor-positive tumour cells: by coupling to a monoclonal antibody against the receptor and by coupling to a high affinity ligand, folic acid. First, antibodies against the folate receptor, including their fragments and derivatives, have been evaluated for tumour imaging and immunotherapy clinically and have shown significant targeting efficacy in ovarian tumour patients.

Folic acid, a high affinity ligand of the folate receptor, retains its receptor binding properties when derivatized via its γ-carboxyl. Folate conjugation presents an alternative method of targeting through folate receptor. This second strategy has been successfully applied in vitro for the receptor specific delivery of protein toxins, anti T-cell receptor antibodies and diagnostic agents for magnetic resonance imaging.

Conjugates to folic acid linked to virtually any molecular complex or molecule of diameter less than 150 nm bind to receptor possessing high binding affinity and enter the cell via receptor mediated endocytosis. Since the same folic acid conjugates could not bind to folate receptor negative cells, folate conjugates show significant selectivity for tumour cells in vivo.

2.9.2.2 Hyaluronic acid as a ligand

Hyaluronic acid (Hyaluronan, HA) is a high molecular weight glycosamino glycan, which is component of the extracellular matrix that is essential for cell growth, structural organ stability and tissue organization. The chemical structure of HA was determined in 1950s in the laboratory of Karl Meyer. HA is a polymer of repeating disaccharide units of

D-glucoronic acid and (1-b-3) N-acetyl-D-glucosamine. The amount of HA in a tissue depends on HA synthesis by HA synthases, internalization by cell surface receptors and extra cellular degradation by hyaluronidases.

HA has a binding affinity to specific cell surface receptors such as CD44, a receptor for Hyaluronic acid mediate motility (RHAMM) and lymphatic vessel endothelial Hyaluronan receptor-I (LYVE-1). The principal receptor of HA is known as CD44, which can regulate cell proliferation and movement. After internalization into the cells, HA is fragmented into endosomes and the fragments of HA are released into the cytoplasm. Several downstream pathways after the activation of CD44 by HA are deregulated in cancer and some of these pathways lead to tumour growth, progression and metastasis. Alterations in both CD44 and HA expressions have been widely observed in tumours from cancer patients and in animal models of tumours. During carcinogenesis, the expression of the standard form of CD44 is up regulated in certain cancers.

HA-chitosan nanoparticles were designed for topical ocular delivery of genes. HA has been commonly used in ophthalmology due to its biocompatible, biodegradable and mucoadhesive properties. Moreover, localization of CD44 has been observed in ocular tissues.

A recent study reported a correlation between CD44 expression and invasiveness of breast cancer cells. Other studies suggested that the molecular sizes of HA fragments play a role in the downstream pathways of HA-responsive cells. Depending upon its size HA exerted differential regulations on wound healing process of fibroblasts. HA mediated targeted delivery systems of nucleic acid based therapeutic moieties need to account for the molecular sizes of HA and different expression levels of HA receptors depending on the progression stage of the cancer.

HA-mediated tumour targeting systems may need to exploit the leaky vasculature of tumour tissues. Although CD44 expression is known to be upregulated in many cancers of epithelial origin, CD44 is also present on normal epithelial cells. Extravasation to tumour tissues might be a pre requisite of expecting the tumour targeting of nucleic acids

by HA-linked delivery systems. One of the limitations in use of HA as a tumour targeted delivery of anti cancer nucleic acid would be the distribution of HA linked systems to skin tissues where the expression of CD44 is high.

HA also exhibits significant cytoadhesive and bioadhesive properties. Bioadhesive microspheres composed of Hyaluronan have been used to prolong the retention time of the drug within the nasal cavity and pulmonary areas.

A temporary widening of the tight junctions of cultured cells, which coincided with an increase in the rate of absorption of the applied drug can be observed in presence of bioadhesive microspheres. It is likely that dry Hyaluronan microspheres took up water from the cells causing them to dehydrate and shrink resulting in separation of the intercellular junctions.

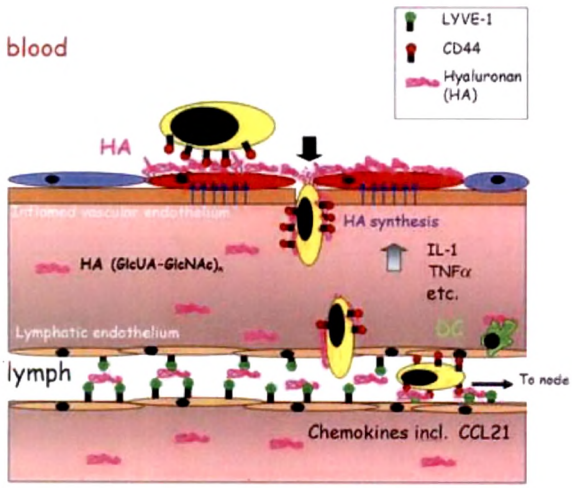

Fig. 2.4- Schematic presentation of interaction of HA and CD44 at cell receptor level.

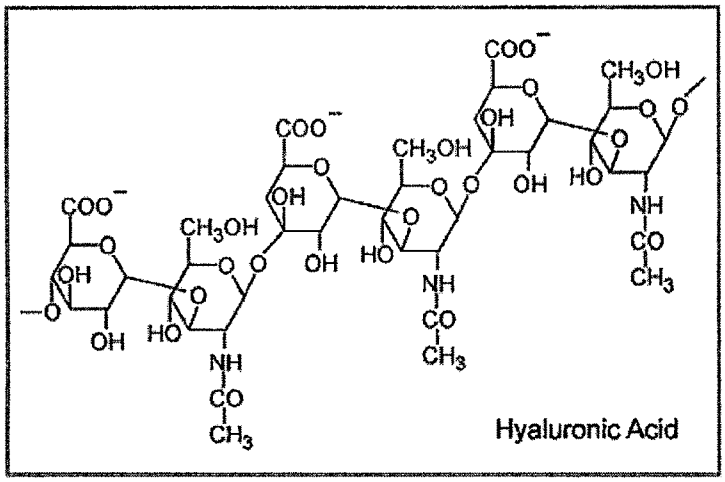

Fig. 2.5- Chemical structure of Hyaluronic acid (Hyaluronan-HA)

2.10 DRY POWDER INHALERS

Inhaled drug delivery systems can be divided into 3 principal categories: pressurized metered-dose inhalers (pMDIs), DPIs and nebulizers, each class with its unique strengths and weaknesses. This classification is based on the physical states of dispersed-phase and continuous medium, and within each class further differentiation is based on metering, means of dispersion, or design. Nebulizers are distinctly different from both pMDIs and DPIs, in that the drug is dissolved or suspended in a polar liquid, usually water. Nebulizers are used mostly in hospital and ambulatory care settings and are not typically used for chronic-disease management because they are larger and less convenient, and the aerosol is delivered continuously over an extended period of time. pMDIs and DPIs are bolus drug delivery devices that contain solid drug, suspended or dissolved in a non polar volatile propellant or in a dry powder mix (DPI) that is fluidized when the patient inhales. The clinical performance of the various types of inhalation devices has been thoroughly examined in many clinical trials, which have been reviewed by Barry and O'Callaghan. Those authors concluded that none of the devices are clinically superior and that device selection should be guided by other factors, such as convenience, cost and patient preference. First approved in 1956, the pMDI was the first modern inhaler device.

With a global market share of about 80%, the pMDI remains the most widely used device. The development of DPIs has been motivated by the desire for alternatives to pMDIs, to reduce emission of ozone-depleting and greenhouse gases (chlorofluorocarbons and hydrofluoroalkanes, respectively) that are used as propellants, and to facilitate the delivery of macromolecules and products of biotechnology. Concurrently, 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, which makes premature deposition in the oropharynx more likely. Thus, pMDIs require careful coordination of actuation and inhalation. Despite enhancements to their design (e.g. use of spacers), incorrect use of pMDIs is still a prevalent problem; 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 was 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 reduces the rate of chemical degradation and the likelihood of reaction with contact surfaces. By contrast, pMDI formulations, which include propellant and cosolvents, may extract organic compounds from the device components.

Most DPIs contain micronized drug blended with larger carrier particles, which prevents aggregation and helps flow. The dispersion of a dry powder aerosol is conducted from a static powder bed. To generate the aerosol, the particles have to be moved. Movement can be brought about by several mechanisms. Passive inhalers employ the patient's inspiratory flow. When the patient activates the DPI and inhales, airflow through the device creates shear and turbulence; air is introduced into the powder bed and the static powder blend is fluidized and enters the patient's airways. There, the drug particles separate from the carrier particles and are carried deep into the lungs, while the larger carrier particles impact in the oropharynx and are cleared. Thus, deposition into the lungs is determined by the patient's variable inspiratory airflow. Inadequate drug/carrier separation is one of the main explanations for the low deposition efficiency encountered

with DPIs. Dose uniformity is a challenge in the performance of DPIs. This is a greater concern with powders than with liquids because of the size and discrete nature of the particulates. Various dispersion mechanisms have been adopted for DPIs. While most DPIs are breath-activated, relying on inhalation for aerosol generation, several power-assisted devices (pneumatic, impact force, and vibratory) have been developed or are currently under development. These devices are being considered for the delivery of systemically active drugs that have narrow therapeutic windows. It is important to note that these "active" inhalers are not subject to the same limitations as passive inhalers and have a different advantage/disadvantage profile. Moreover, it has been suggested that if shear and turbulence could be standardized by using a dispersion mechanism that is independent of the patient's breath, high delivery efficiency and reproducibility might be achieved. Thus, an active inhaler might provide formulation-independent delivery.

2.10.1 Powder and Aerosol Physico Chemical Characterization

The character of particulate systems is central to the performance of DPIs. Powders present unique design challenges. Powders are 2-phase gas-solid systems. When powders are static, they behave as solids; when they flow, they resemble liquids, easily assuming the shape of the containing vessel. When a powder is dispersed in air, as is the case after actuation of a DPI, in many ways it conforms to its carrier gas (unlike gases or vapors, pharmaceutical powders are non equilibrium systems). Whereas gas and liquid behavior is understood and accurately predicted by equations derived from first principles, physical equations governing powders are often empirical or rely on assumptions that are only approximations to real systems, such as homogeneity in size and shape of particles. As a consequence, equations describing the behavior of solids are less predictive than their fluid counterparts.

Powder features such as the physicochemical properties and morphology of its constituent particles and the distribution of particle sizes, contribute to variability. Unlike liquid solutions or gas mixtures, powders are never completely homogeneous (at primary particulate scale) and segregation by size, which is a function of external forces, is always

a potential problem. The aerodynamic behavior, which has a profound effect on the disposition of drug from a DPI, is particularly sensitive to powder properties.

2.10.1.1 Moisture Content and Hygroscopicity

Hygroscopicity is the intrinsic tendency of a material to take on moisture from its surroundings. The hygroscopicity is affected by the crystallinity of the material and the morphology of the particles. Hygroscopic drugs present a greater risk of physical and chemical instability. Moisture uptake and loss due to changes in relative humidity can result in local dissolution and leading to irreversible aggregation through solid bridge formation which can adversely affect aerosol generation and lung deposition. Hygroscopicity can also alter the adhesive and cohesive properties, or, in more extreme situations, substantially increase particle size. Hygroscopic growth has implications for the equilibrium moisture content of the particles in the dosage form prior to aerosol generation; it can cause chemical or physical instability of the product. For aerosols, the physical instability is more important, because agglomeration may be irreversible and lead to an inability to generate aerosol particles of respirable size. As aerosol particles enter the lungs, they experience a high-humidity environment (99.5% relative humidity at 37°C). Although they may not reach equilibrium during transit, susceptible aerosol particles may be subject to hygroscopic growth, which increases particle dimensions and affects lung deposition. Hygroscopic growth can be prevented by coating the drug particles with hydrophobic films. However, no such approach has been successfully implemented in a marketed formulation. The equilibrium moisture content of a drug and excipient must be determined over a range of relative humidities, so that storage conditions can be defined and other protective measures considered. Excipients that modify the hygroscopic properties of a drug may need to be considered.

2.10.1.2 Particle Size

Particle size is the single most important design variable of a DPI formulation. Methods for determining particle size and distribution use various geometric features or physicochemical properties. Among these, aerodynamic diameter is the most relevant to

lung delivery and ultimately to therapeutic effect. There is substantial literature from the fields of industrial hygiene, environmental and occupational medicine, and pharmaceutical sciences that links aerodynamic size and size distribution to the probability of deposition in specific lung sites. The statistical basis for these relationships in terms of variability in airways geometry and lung physiology, both between individuals and within an individual, has been sufficient to allow the development of semi-empirical models correlating particle size with lung deposition.

2.10.1.3 Aerodynamic Diameter and Dynamic Shape Factor

Aerodynamic diameter is the most appropriate measure of aerosol particle size, because it relates to particle dynamic behavior and describes the main mechanisms of aerosol deposition; both gravitational settling and inertial impaction depend on aerodynamic diameter. To reach the peripheral airways, where drug is most efficiently absorbed, particles need to be in the 1–5 μm aerodynamic diameter range. Particles larger than 5 μm usually deposit in the oral cavity or pharynx, from which they are easily cleared. In contrast, particles smaller than 0.5 μm may not deposit at all, since they move by Brownian motion and settle very slowly. In a series of studies, the optimal particle size of aerosol particles was examined for several different therapeutic agents in patients with different disease states. Although some differences due to patient lung function were noted, the optimal size was always in this 1–5 μm range. The aerodynamic diameter, Dae, is defined by the diameter of an equivalent volume sphere of unit density Deq with the same terminal settling velocity as the actual particle. For particles larger than 1 μm, the following expression describes the relationship between pharmaceutical powders are rarely spherical, and shape factors are dimensionless measures of the deviation from sphericity. The dynamic shape factor is the ratio of the actual resistance force experienced by the non spherical falling particle to the resistance force experienced by a sphere having the same volume. It is the aerodynamic diameter that determines lung disposition, irrespective of geometric particle size (to a certain point). The aerodynamic

diameter can be decreased by decreasing the particle size, decreasing particle density, or increasing the dynamic shape factor.

2.10.1.4 Fine-Particle Fraction

"Fine-particle fraction" is the percentage of particles in the fine-particle range (1–5 μm). "Fine-particle mass" is the total mass of the particles that are in the fine-particle range. The fine-particle component of aerosols is usually defined as the percentage of particles that are smaller than 5 μm aerodynamic diameter, or, in the case of certain particle-sizing instruments, a cut-off diameter that is close to 5 μm. Quite often this may be in the 6–7 μm range. The danger of adopting these values as definitive measures of equivalency is associated with the effect of particle size on deposition.

2.10.1.5 Polydispersity

For drug delivery it is the convention to consider the mass associated with each particle size as the frequency term in the distribution, since this relates directly to dose. Conventional statistical properties apply to populations of particles (i.e. mode, mean, and median). It is usual to define the central tendency of numbers of aerosol particles by the mass median aerodynamic diameter, which reflects the particle size that divides the distribution in half as a function of mass.

When considering particle size, the degree of Polydispersity (i.e., the range of particle sizes around the mode) is also important. The simplest and preferred system exhibits a single mode. However, many pharmaceutical aerosols will exhibit more than one mode. It is conceivable that completely different aerosol distributions (e.g., small median size with narrow distribution or large median size with broad distribution) could give exactly the same fine particle fraction. However, within the fine-particle fraction, the aerosol would exhibit different sizes, leading to differences in regional lung deposition, resulting in variations in therapeutic effect. Thus, degree of dispersity is an important consideration for both quality and efficacy of pharmaceutical aerosols. The nature of the aerosol

distribution must be established accurately if its implications for deposition and efficacy are to be understood.

2.10.2 Particle Sizing Techniques

Several techniques are available for determining particle size distributions. The aerosol sizing techniques can be classified as (1) inertial methods, (2) light-scattering methods, or (3) imaging methods.

Cascade impactors including multi-stage liquid impingers, are the most widely used instruments for sizing aerosols; they are recommended by both the United States and the European pharmacopeias.

Their utility stems from the fact that they directly measure aerodynamic size, rather than equivalent volume diameter (based on cross-sectional area) like the other methods. The theory of cascade impactor operation has been described in depth elsewhere. Briefly, cascade impactors contain several stages, with orifices of decreasing size, stacked on top of each other. When the aerosol is drawn through the impactor, the particles deposit on different stages, based on their inertia. After each run, the impactor is disassembled and the mass of particles deposited on each stage is determined, mostly via analytical methods (dissolution in solvent, followed by chromatography or ultraviolet absorbance).

A cut-off diameter is associated with each stage of the impactor. This diameter varies with airflow, so the impactor must be calibrated for different flow rates. This airflow dependence allows investigation of the effect of different inspiratory flow rates on deposition. Particles passing through the electrical low-pressure impactor are charged before traversing the cascade of stages. Their impact on the stages produces an electrical current that is detected and converted into particle-size data that can be interpreted immediately. A limitation of the electrical low-pressure impactor is that it is not suitable for particles larger than 20 μm so it cannot be used to size carrier particles which limits its utility for sizing pharmaceutical aerosols. Based on a PubMed search, reference to electrical low-pressure impactors in medical/pharmaceutical journals is limited to a single

publication, in which an electrical low-pressure impactor was used for sizing sub-micron size pMDI particles. However, the electrical low-pressure impactor has great potential to simplify the aerosol sizing process and is likely to make an impact in the field in the future.

Light Scattering and Laser Diffraction.

An expanded laser beam is passed through a sample that is being drawn through a measuring zone. Different size particles diffract the light at different angles. A computer algorithm, which differs between manufacturers, interprets the diffraction pattern and calculates a particle size distribution. The algorithms are based on Fraunhofer or Mie theory, from which the particle sizes are determined. Since the algorithms differ among the different instruments, comparisons are difficult, particularly for the majority of pharmaceutical particles, which deviate from sphericity.

Surface Area and Morphology

Particle surfaces are important elements in particle interactions, stability and ease of dispersion. Since aerosol particles are small, the total surface area of a powder is very large. A large surface area renders the particles subject to greater potential for charging and moisture uptake. In addition, the size of the particles renders them more susceptible to the influence of van der Waals forces.

Forces of Interaction

Particle separation is the most important performance characteristic for effective aerosol generation. To separate particles, specific forces of interaction must be overcome. There are 4 major forces of interaction between particles: mechanical interlocking due to surface asperities, capillary forces from the presence of water, electrostatics arising from the insulating nature of the material, and van der Waals forces from the fundamental electromagnetic nature of matter. Much has been written on the subject of particle interactions, both from a solid state physics and a therapeutic aerosol standpoint. On a large scale, physical interactions are barriers to aerosol generation. In this case,

mechanical interlocking due to surface features or roughness is a prominent mechanism preventing particle dispersion. Temperature and humidity cycling, or poor drying may also result in solid bridging, through crystallization/recrystallization phenomena at the particle surfaces. The presence of moisture, even in small quantities, will also bring about capillary forces. The magnitude of these forces is related to the diameter of the pores between particles and the interfacial tension due to hydrogen bonding of water. Controlling moisture content will aid in reducing capillary forces, but care must be taken to avoid increasing the surface charge of the particles.

2.11 FORMULATION OF DPIs

The particle size distribution affects the deposition of drug in the respiratory tract. However, before drug can be delivered to the lungs, drug particles must leave the DPI and separate from each other and from other components in the formulation. Thus, a DPI formulation must undergo flow, fluidization and segregation. However, micron sized particles, particularly those resulting from high-energy operations such as jet milling, have high surface areas and surface energies, which result in poor flow and a high tendency to aggregate. Formulation strategies aim at alleviating these problems.

2.11.1 Excipients

One way to improve the non pharmacological properties of a drug is through the addition of excipients. In general, excipients are used to enhance the physical or chemical stability of the active pharmaceutical ingredient, its mechanical properties, and/or its pharmaceutical properties, such as dissolution and permeation. In DPI formulations, excipients function first and foremost as carrier particles. Usually, no more than a few milligrams of drug need to be delivered, and excipients provide bulk, which improves handling, dispensing, and metering of the drug. Excipients also reduce drug cohesiveness by occupying the high-energy sites of the drug particles. The primary function of the lungs is respiration. To fulfill this purpose, the lungs have a large surface area and thin membranes. Unlike the gastrointestinal tract, the lungs have limited buffering capacity. Many compounds that could enhance drug delivery outcomes also have the potential to

irritate or injure the lungs. Consequently, the array of potential excipients is limited to compounds that are endogenous to the lung and can easily be metabolized or cleared. Currently, lactose is the only excipient used in DPIs marketed in the United States. The reasons for this are as much historical as they are physico chemical/pharmaceutical in nature. Lactose had long been used as an excipient in oral dosage forms before being deployed in DPIs. It had an established safety and stability profile, manufacturing process with tight controls over purity and physical properties, and was available and inexpensive. Lactose is highly crystalline and has the smooth surfaces and satisfactory flow properties desirable for a DPI carrier particle. Lactose is less hygroscopic than other sugars. Lactose is quite versatile; several manufacturers offer excipient-grade lactose of various sizes and morphologies. One of the drawbacks of lactose is that it is a reducing sugar, which makes it incompatible with drugs that have primary amine moieties. Other sugars, such as mannitol have been shown to be feasible alternatives to lactose, and it is expected that these sugars will eventually find their way into approved products. Glucose is already used in DPIs in Europe. Phospholipids such as phosphatidylcholine and cholesterol have also been used in experimental liposomal formulations. Several other materials have been included in experimental DPI formulations, with various objectives and varying success. Excipients can makes up over 99% of the product by weight, making them crucial determinants of overall DPI performance. Despite the apparent lack of choices, the excipient must be carefully selected; physicochemical properties such as size and morphology profoundly affect the performance of the formulation. The adhesive forces must be carefully considered; inadequate separation of drug and carrier is the main reason for deposition problems. The formulator may also choose to modify the excipient before combining it with the drug. It should also be noted that excipients are not always required; the Pulmicort (budesonide) Turbuhaler (AstraZeneca, Wilmington, Delaware) is an example of an excipient-free formulation.

Porous or hollow particles exhibit very different equivalent volume diameters from their aerodynamic diameters, because of the density terms, as described by the Stokes equation. Particles can be made in the respirable aerodynamic diameter range, even as

their geometric particle size is on the order of 20 μm. This offers some important advantages in the dispersion of these particles, due to the reduced van der Waals forces, which reduces their tendency to aggregate and makes them more responsive to shear in an airflow path. However, there is a limit to how much such an approach can be used, because the peripheral airways of the lungs are very small. Consequently, beyond a particular geometric size, penetration to the periphery would not be possible. In addition, low-density particles carry little mass in a unit volume. Therefore, the limits on dose delivery must be considered carefully. With these caveats, for potent, low-dose drugs these particles can be excellent delivery systems.

Loose agglomerates have been used as a means of stabilizing powder aerosols, so that, upon the introduction of energy from the patient's breath or some active source, they readily disperse into small particles for inhalation. These agglomerates can consist of particles of disparate sizes, as is the case when drug is prepared with large carrier particles, or particles of similar sizes prepared by unique methods of formation that result in ease of dispersion (Turbuhaler and Twisthaler formulations).

2.12 PHARMACEUTICAL PROCESSING

The processes involved in powder formulation have been extensively reviewed in the pharmaceutical technology/engineering literature. After drug and excipient(s) have individually been brought to their desired forms, they are combined in the blending process. The importance of the blending process can be easily overlooked. However, it is a critical step in the manufacture of a DPI product and is in fact subject to substantial optimization work during development. When mixing powders with different properties, particle sizes, and ratios, as is the case with DPI formulations, inadequate mixing can cause poor dose uniformity. In many cases, inadequate mixing cannot be overcome simply by increasing the mixing time. Mixer selection, rotation speed, capacity, and fill level are all subject to optimization, as they can all affect the blend homogeneity. Blending conditions also affect inter particulate forces, which are a primary determinant of the fine-particle fraction. Different powders may have different mixing requirements,

depending on the forces present between the various particles. For low concentration (drug-carrier ratio) blends, geometric dilutions are necessary pre blending steps. The flow properties of the components of the powder blend will play an important role in the efficiency of blending and, ultimately, in aerosol dispersion. Powder flow properties have been studied for some time, and methods have been adopted for their characterization, including bulk and tapped density and angle of repose. Powder sampling is an important prerequisite for accurate characterization. Blending validation is an important activity required by Good Manufacturing practices in the United States Code of Federal Regulations. However, taking blend samples at different times to determine the uniformity of the blend is associated with several difficulties. New techniques are emerging that can determine the blend homogeneity without removal of samples from the mixer; techniques such as near-infrared and Fourier transform-infrared analysis can determine blend uniformity by nondestructive acquisition of infrared spectra. After the formulation has been blended, it is filled into capsules, multi-dose blisters, or reservoirs for use with the inhaler device. The filling process is automated and depends on the nature of the metering system. In order to maintain its physical and chemical integrity and dispersibility, the product must be stored appropriately. Storage conditions, such as temperature and relative humidity profoundly effect DPI stability and performance and hence, permissible storage conditions need to be determined.

Interest in DPIs has increased in the last decade, in response to the need for alternatives to propellant-driven devices and new approaches to the delivery of potent new chemical entities of biological origin. The number of diseases that are being considered candidates for aerosol therapy has increased substantially. Until recently, asthma was the only clear example of a disease that could be treated via aerosol delivery to the lungs. We now consider it possible to treat not only asthma and chronic obstructive pulmonary diseases but also systemic disorders such as diabetes, cancer, neurological diseases (including pain), and other pulmonary diseases such as cystic fibrosis and pulmonary infectious diseases. DPIs offer unique opportunities and unique challenges. The opportunity to use solid-state physics and chemistry to prepare stable, dispersible particles for aerosol

delivery to the lungs is clear. The challenges relate to the unique formulation strategies required and the susceptibility of dry powders to forces of interaction caused by their surface and bulk energetics, which can inhibit their dispersion and limit aerosol delivery and, therefore, efficacy.

2.13 A549 CELL PROFILE

(Human, Caucasian, lung, carcinoma)

Morphology: Epithelial

Growth properties: Adherent

Organism: *Homo sapiens* (Human)

Source: Organ: Lung; Disease: Carcinoma

Cellular Products: Keratin

Isolation Date: 1972.

Reverse Transcriptase : Negative

DNA profile

Amelogenin:X,Y
CSF1PO:10,12
D13S317:11
D16S539:11,12
D5S818:11
D7S820:8,11
THO1:8,9.3
TPOX:8,11
vWA: 14

Cytogenetic Analysis

This is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. Cells with 64 (22%), 65, and 67 chromosome counts also occurred at relatively high frequencies; the rate with higher ploidies was low at 0.4%. There were 6 markers present in single copies in all cells. They include der (6)t(1;6) (q11;q27); ?del(6) (p23); del(11) (q21), del(2) (q11), M4 and M5. Most cells had two X and two Y chromosomes. However, one or both Y chromosomes were lost in 40% of 50 cells analyzed. Chromosomes N2 and N6 had single copies per cell; and N12 and N17 usually had 4 copies.

Isoenzymes: G6PD, B

Comments: This line was initiated in 1972 by D.J. Giard, et al. through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. Further studies by M. Lieber, et al. revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway. The cells are positive for keratin by immunoperoxidase staining.

Propagation

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C

Subculturing Protocol

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels. Cultures can be established between 2 X 10(3) and 1 X 10(4) viable cells/cm2. Do not exceed 7 X 10(4) cels/cm2.

6. Incubate cultures at 37°C.

Interval: Maintain cultures at a cell concentration between 6 X 10(3) and 6 X 10(4) cell/cm2.
Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:8 is recommended
Medium Renewal: 2 to 3 times per week

Preservation

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Doubling Time: about 22 hours

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2004
Recommended serum: ATCC 30-2020

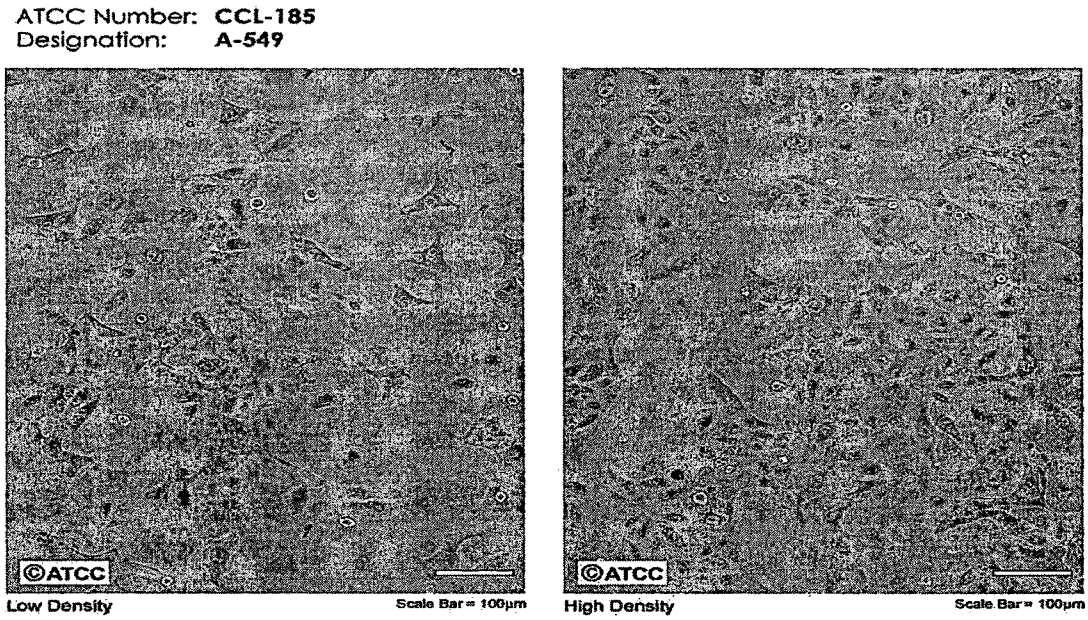

Fig. 2.6: Alveolar epithelial cells (A549 cells)

2.14 REFERENCES

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