2. LITERATURE REVIEW

2.1 The concept of nanocrystals as delivery system of lipophilic drugs

2.1.1 Definition and history

Drug nanocrystals are drug nanoparticles constituted of 100% drug without any matrix material, with a mean particle size below 1 µm, typically somewhere between 200 and 500 nm (Keck and Muller 2006). Drug nanocrystals are formulated as nanosuspensions, having the drug nanocrystals dispersed in a dispersion medium composed by surfactants or sterically stabilising polymers as primordial excipients to avoid nanocrystals aggregation.

Mainly three production technologies for drug nanosuspensions are widely used:

- Pearl/ball milling
- High pressure homogenisation
- Microprecipitation

In 1992 Liversidge *et al.* described dispersions of drug particles with size diameters between 150 and 400 nm that could be stabilized with surfactant adsorbed on the surface of the particles (Liversidge, Cundy et al. 1992). The process to achieve this particle reduction was pearl milling. The milling pearls in this kind of systems are made of glass, zircon oxide or especially hard polymers. This technique is currently used to prepare the so called NanoCrystals™ currently produced by the company élan Drug Technologies. The drug needs to be milled from hours to days depending on the hardness of the drug.

The high pressure homogenisation technique using piston-gap homogenisers was first described by Müller *et al.* in 1994 (Müller, Becker et al. 1994). See section 3.2.1 (page 51) for details of the process. The production of nanocrystals so called DissoCubes® using water based dispersion media is a technology acquired by SkyePharma PLC in 1999 (Müller, Becker et al. 1999) being licensed out to Baxter International Inc.

In 2000 a new generation of nanoparticles arose using high pressure homogenisation to reduce the size of particles in a non-aqueous or water content-reduced dispersion media (Müller, Mäder et al. 2000). The new pure nanocrystals are so called Nanopure®.

Finally, in 2002 the company Baxter International Inc. has developed a combined technology so called NANOEDGE^{TM}. The production process involves mainly two steps, the first is a precipitation step and the second step consists on adding energy to the pre-suspension to form particles having an average effective particle size of 400 nm to 2 microns. The last step occurs by an energy input technique, e.g. high pressure homogenisation of the particles using microfluidisation or piston-gap homogenisers. The process is called “microprecipitation” (Kipp, Wong et al. 2003).

2.1.2 Saturation solubility

In order to be absorbed, a drug will pass a dissolution process in the fluid at the site of absorption. Class II drugs show poor solubility (and related low dissolution velocity) and good permeability, which means that their bioavailability problems can be overcome when improving their solubility (Amidon, Lennernäs et al. 1995). The saturation solubility is a temperature dependent constant for each substance, but for particles below approximately 1-2 µm, an increase in saturation solubility will occur (Bisrat and Nyström 1988; Mosharraf and Nystrom 1995). Due to an increase in the curvature of the particle surface the dissolution pressure of the substance will increase according to the Kelvin equation (Müller and Böhm 1998). The increased solubility (or saturation solubility) of the drug is correlated to a faster dissolution velocity by the Noyes-Whitney equation (see below).

2.1.3 Reduction in particle size and dissolution velocity

By reducing the size of the drug particles to nanocrystals, one increases the surface area A to be in contact with the dissolution medium. As a result, the dissolution velocity *dm/dt* will also increase. This will be directly correlated to a reduction in *h*, which is the streamline layer in contact with the solid when the solvent is moving in streamline flow. As a consequence, a concentration gradient occurs along this layer (Carless 1977).

The dissolution of a drug is described by the general Noyes-Whitney equation:

$$\frac{dm}{dt} = \frac{A \cdot D\,(C_s - C_x)}{h}$$

where:

$\frac{dm}{dt}$ = dissolution rate

A = surface area of dissolving solid

k = dissolution rate constant

C_s = concentration of drug in the saturated diffusion layer

C_x = concentration of drug in the dissolution medium at time x.

h = thickness of the streamline layer

High pressure homogenisation will reduce the thickness of the diffusion layer towards zero, which will increase the rate of dissolution of the nanoparticles.

2.1.4 Overview of current applications

2.1.4.1 Oral delivery

Oral administration is probably the route most widely accepted by patients and at the same time, the faster way to the market compared to e.g. parenterals. Formulations containing nanocrystals for this application have already been placed on the market. Examples of these products are the oral tablets Rapamune®, commercialized by Wyeth Pharmaceuticals in 2000; the oral capsules Emend® (Merck, 2003); TriCor® (Abbott, 2004) and the oral suspension of megestrol Megace® ES (Par Pharmaceutical Inc., 2004).

2.1.4.2 Intravenous delivery

Nanosuspensions are an attractive formulation for the intravenous application, as their particle size reduces the risk of emboli due to capillaries blockage. A successful approach for the intravenous therapy of *Mycobacterium avium* infection was reported with the use of clofazimine nanosuspensions produced by high pressure homogenisation (Peters, Leitzke et al. 2000). The company Baxter developed aqueous itraconazole nanosuspension for the parenteral application (Rabinow 2004; Neuberger and Wong 2005). In a rat model, they have shown that the new formulation is well tolerated and show less nephrotoxicity (Rabinow and et.al. 2003). Atovaquone nanosuspensions have shown an excellent *in vitro* effectivity in a murine model of reactivated toxoplasmosis (Schöler, Krause et al. 2001) showing the capacity of the drug nanocrystals to pass through the BBB and target the brain.

Although several parenteral drugs are nowadays in development phases, none of them has still reached the market.

2.1.4.3 Inhaled delivery

The use of nanocrystals for pulmonary drug delivery has been proposed in several investigations. Müller *et al.* in 2002 have proposed the use of budesonide nanocrystals produced by high pressure homogenisation as a nebulizer suspension (Jacobs and Müller 2002). In 2004, Kraft *et al.* in collaboration with the company Sheffield Pharmaceuticals (Rochester, NY) have published the benefits of using budesonide nanosuspension in a primary study with healthy volunteers. In 1999 Ostrander et al. (élan Drug Technologies) have in vitro investigated the use of beclomethasone dipropionate as a colloidal dispersion of nanocrystals to be nebulized in ultrasonic nebulizers (Ostrander, Bosch et al. 1999). Although the use of nanocrystals for the pulmonary delivery of poorly soluble drugs is an attractive field and initial investigations show promising results, no product had still reach the market with this administration route.

2.2 Pulmonary anatomy and physiology

2.2.1 Anatomy and passage of particles through the airways

The human respiratory tract has an extensive blood supply and its total surface area is about 75-140 m^2. Most of the surface area resides in the alveolated regions of the deep lung, which also contains a rich capillary network to assure efficient gas exchange during the breathing process (Gonda 1988).

The respiratory tract can be divided into upper and lower parts by the lower border of the cricoid cartilage. A review of the anatomical details of this region based on previous publications (Clarke 1984; Hickey and Thompson 1992; Phalen, Yeh et al. 1995) is given in the following paragraphs.

2.2.1.1 The nose

The anterior nostrils lead from the outside to the beginning of the ciliated mucosa at the anterior ends of the nasal sputum and turbinates. The main channel extends backwards 6-8 cm to the posterior ends of the turbinates and septum. The entrance or vestibule is lined by skin with sebaceous glands and contains long strong hairs,

which also help to filter the air stream. The mucus membrane of the nasal cavity is ciliated, highly vascular and rich in mucus glands.

2.2.1.2 The nasopharynx

The nasopharynx lays 12-14 cm from the external nostrils where the septum ends and where there is a transition from columnar ciliated to squamous epithelium extending to the larynx. The nasopharynx ends at the lower border of the soft palate. The upper airways have specific functions, because it helps to filter the airflow, heat and humidify the airflow and it is also important in olfaction, vocal resonance with speech and nasal reflexes. In this region of the respiratory system, clearance is carried out through the sweeping of nasal secretions backwards by mucociliary action. At the mid-nasal passage the temperature of inspired air rises to about 30 °C and 33 °C in the nasopharynx with a relative humidity of 100% at each site.

2.2.1.3 The larynx

The laryngeal dimensions in humans are about 4.4 cm length x 4.3 cm across x 3.5 cm in interior-posterior extent, being relatively smaller in women. The cavity of the larynx extends from the pharynx to the commencement of the trachea and the border of the cricoid cartilage. The larynx has also several important functions acting as the respirarory channel and airflow regulator, it is a receptive field for reflexes (e.g. cough reflex) and also acts as a sphincter in fixation of the thorax during cough and expectoration and with protective closure on swallowing and vomiting. It has also importance in phonation and speech modulation.

2.2.1.4 The trachea and the lower airways

The trachea has 16-20 cartilaginous rings, 3-4 mm wide and posterior longitudinal smooth muscle, extending from the larynx to the bifurcation (carina). The average adult length is 11 cm (range 9-15 cm) with a lateral diameter of 20 mm and anteroposterior of 15 mm. The diameters increase during inspiration and decrease during expiration, involving the membranous posterior wall of the trachea and main bronchi. The carina is usually sharp and mobile on deep breathing or coughing, and lies 25-27 cm from the upper incisors.

In the lower airways, the mucosa is pseudostratified, ciliated columnar with numerous goblet cells resting on a broad basement membrane with mucus glands containing mucus and serous elements lying in the submucosa and contributing to the mucus

"blanket" overlying the cilia. This anatomy facilitates in healthy individuals the clearance of most of the deposit particles within 24 hours by mucociliary action to the throat for swallowing. Relatively soluble material may quickly enter the bloodstream. The trachea divides into right and left main bronchi which themselves give off three lobar bronchi on the right (upper, middle and lower lobes) and two on the left (upper and lingula and lower). Both main and lower lobe bronchi lie outside the lung substance and may be termed the "large bronchi" being 7-12 mm in diameter. The lower bronchi divide into about 20 bronchopulmonary segment bronchi -"the medium bronchi" 4-7 mm in diameter. Bronchioles have no cartilage in their walls and are 500-800 μm in diameter. Circular and diagonal muscle fibers normally surround the ligaments inside the cartilage, contracting, constricting and shortening the airway. Lymphoid tissue is found in the mucosa often in solitary nodules and particularly at points of bifurcation. The number of alveoli in the lungs increases from about 24×10^6 at birth to 280×10^6 at age eight years after which the number remains virtually constant. Their size is about 200-300 μm.

2.2.2 Physiology

The tissue at the nose, larynx, trachea, bronchi and bronchioles is characterized by the presence of cells with numerous cilia and by individual cells and glands that secret the components to make up mucus. Movement of the overlying mucus will displace this viscoelastic fluid toward the glottis at rates up to several hundred cycles per minute where it is swallowed. This tissue is responsible for sweeping surfaces of the airways free of particulate contamination.

The alveolus is a polyhedral structure having one face open to the airway. Their walls are formed by very thin alveolar epithelial cells, being these of more than one type. At its thinnest portions, the type I alveolar epithelial cell is about 100 nm or less in thickness. These cells lie on top of a basement membrane about 20-40 nm thick. These cells are responsible for the alveolar gas exchange. Another basement membrane supports the blood capillary endothelial cells which join to form the capillary wall. These cells are similar in shape and size to the thin alveolar cells. Another cell present in the alveolus is the type II epithelial cell, a roughly cube-shaped cell. The main function of this cell is the production and secretion of surfactant, a surface-tension-lowering agent that reduces the tendency alveoli have for collapsing. Surfactant consists of lipid-rich lipoproteins with phosphatidylcholine

with a high dipalmitoyl. The content 85-90% of isolated surfactant is lipid with 95% phosphosterols with colesterol as the main neutral component (Taylor and Kellaway 2001). The type II cells may serve as precursors of the type I cells during lung growth and repair. Other cells present in the alveolar region include the macrophages, alveolar brush cells (type III) and interstitial cells. Macrophages are found on the surfaces of the alveoli in the deep lung, but they are not a fixed part of the alveolar epithelial wall. Their main function is to maintain the sterility of the lung by virtue of their ability to engulf and kill infectious microorganisms and foreign particles with mechanisms that seems to involve chemotaxis and phagocytosis.

The lymphatic system of lungs also participates in the maintenance of homeostasis, respiratory defences and in the clearing of inhaled toxicants and particular matter.

During mouth breathing, as is the case with oral inhalation of therapeutics (e.g. by means of nebulizer devices), the collection of larger particles in the nose is not possible. In consequence, the larger particles tend to deposit in the tracheo-bronchial region with higher efficiency.

The respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli constitute the gas exchange region. For particles to reach and deposit in this region, they most contact with deep lung surfaces by settling, diffusion or interception with them. Clearance from this region is not totally understood but the following mechanisms have been proposed:

- Dissolution of relatively soluble material and absorption into the systemic circulation
- Direct passage of particles into the blood
- Phagocytosis by macrophages
- Transfer of particles to lymphatic channels, vessels and nodes.

2.3 General aspects related to pulmonary drug delivery

Due to the large surface area offered (especially in the alveoli) and the highly vascularized thin epithelium, the lung has been always attractive for the systemic administration of drugs and more recently, also for the local therapy of lung diseases. The relatively recent peak of the development of formulations to be delivered to the lungs is related to the advances in the design of new systems such as pMDI (pressurized Metered Dose Inhalers), DPI (Dry Powder Inhalers) and nebulizers. The main challenge in the design of formulations to be delivered to the lungs is the

incorporation of the drug in particles with an appropriate aerodynamic size distribution. O' Callaghan and Barry (1997) discussed the deposition profiles for an inhaled nebulizer cloud as predicted from the model of Rudolf and coworkers (Rudolph, Kobrich et al. 1990) which assumes oral breathing and healthy subjects. They concluded that oropharyngeal deposition decreases with decreasing median droplet diameter, falling from 60% of the inhaled dose at 10 μm to virtually zero at 1 μm. Central airway deposition peaks at 5-7 μm and peripheral airway deposition at 2-3 μm. However, it must be considered that usually the patient's respiratory system status in disease is an important factor that affects directly the behavior of the particles once inhaled (O' Callaghan and Barry, 1997). Particles from 0.1 to 0.5 μm are not deposited in the respiratory tract but exhaled. As the aerodynamic diameter refers not only to the geometric particle diameter, but also to the particle mass density, larger but porous particles have been also proposed as an option for targeting the alveolar region (Edwards, Hanes et al. 1997; Steckel and Brandes 2004). As the aerosol cloud passes from the generator into the airways, the particle size may change by evaporation of the volatile components (solvents) on the surface of the droplets and by condensation of water vapor from the humid airways upon the particles or droplets containing the drug (Gonda 1988). Even if particles have been successfully deposited in the respiratory system, they still need to release the drug substance and survive the clearing mechanisms before they can achieve their therapeutic effect. Therefore, the development of effective pulmonary delivery systems that can reach the alveoli is still a challenge and attractive approach in the treatment of respiratory and systemic disorders. Systemic administration of drugs through the lungs is attractive for substances that suffer from metabolic breakdown in the gastrointestinal tract. Also large molecules, such as peptides and proteins, that cannot pass the absorbing membrane after oral administration (e.g. insulin) are potential substances for this route of administration.

2.3.1 Market perspective

Since the mid-twentieth century, with the growth of high-technology medical engineering, pulmonary drug delivery has grown from a seed concept to a multi-billion dollar industry. In 2001, sales of products using pulmonary delivery systems reached $8 billion (Minter 2003) with the U.S. at 47%, Europe at 34% and Japan at 10%. This was confirmed by analysis from Frost & Sullivan, who published that USA

emerging pulmonary drug delivery technologies markets reveals this industry generated revenues totalling $ 2.30 billion in 2002.

2.3.2 Mechanisms of aerosol deposition in the lung and the aerodynamic size distribution of inhaled particles

The desired site of deposition for therapeutic aerosols depends on the disease itself. The respiratory has a natural barrier to intercept the majority of inhaled aerosols before they penetrate and perhaps damage the peripheral tissues of the lung (Dennis and Hendrick 1992). The size of the particles inhaled will determine their fate and their mechanism of clearance. The larger the particle's aerodynamic diameter, the more readily it will be influenced by gravity and thus removed from the air stream (Dolovich 1989). According to Gonda (1988) and Lippmann (1977), aerosol particles produced by nebulization, will follow one of the mechanisms explained in the next paragraphs, depending on their aerodynamic properties (e.g. size, density, velocity). The Figure 2-1 esquematically represents the main possible mechanisms of deposition followed by a particle inhaled.

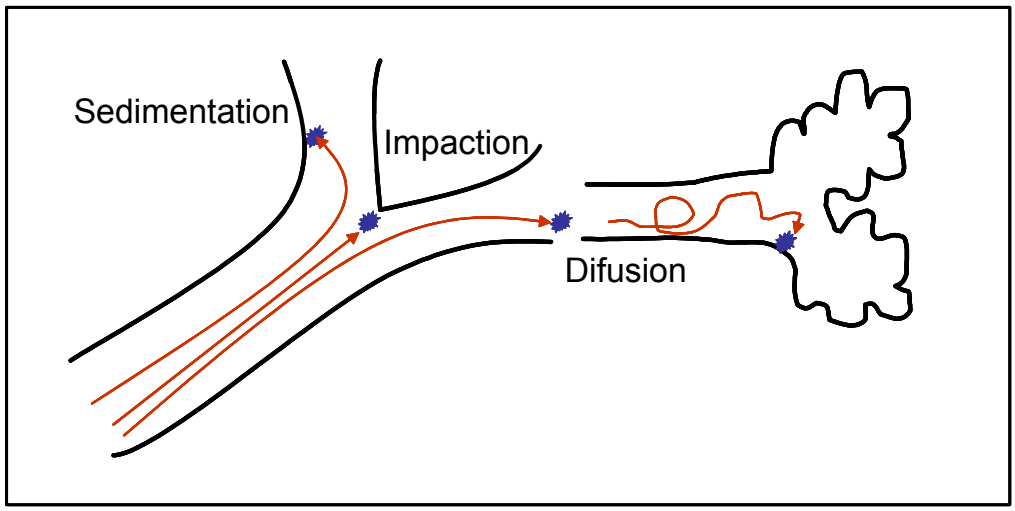

Figure 2-1: Main mechanisms of aerosol deposition in the lung

2.3.2.1 Inertial impaction

It occurs predominantly in the extrathoracic airways and in the tracheobronquial tree. Further bifurcations in the trachea towards left and right tracheobronchial regions will affect the speed of the air stream on its way to the alveolar region (from several m/s

to less than 1 mm/s). In consequence, impaction is not of relevance in the lower airways. A particle carried by a stream of gas exhibits a momentum, which is the product of its mass (inertia) and velocity. When the gas stream encounters an obstacle or a bend, the direction of the gas flow changes. The inertial force of the particle resists this change, causing the particle to fly some distance along the original direction of motion. Therefore, the particle with a high momentum may impact on the surface of the object in front of it, rather than follow the gas streamlines. When oral inhalation occurs, the air stream encounters the first sharp bend (90°) in the pharynx. Particles with a Mass Median Aerodynamic Diameter (MMAD) > 10 μm have a high probability to impact in this area due to high particle mass, according to what was explained above (Gonda 1988). The porous particles with diameters > 10 μm, have more probabilities of success continuing their way, as they are hollow and exhibit lower mass and density (0.03-0.2 g/cm^3 instead of 1 g/cm^3).

2.3.2.2 Sedimentation (settling)

Particles and droplets suspended in gas are subject to the vertical force of gravitation, which acts upon them in all parts of the respiratory tract. The settling velocity increases with the square of the aerodynamic diameter. Sedimentation is therefore, an important mechanism of deposition in the lower airways for particles, which escape the capture by impaction (0.5 μm < aerodynamic diameter < 3 μm).

The sedimentation velocity of particles with aerodynamic diameter < 0.5 μm is so low that they would not deposit in the lower airways by settling, unless they were held there over a prolonged period of time (Gonda, 1988).

2.3.2.3 Diffusion (Brownian motion)

As a resultant effect of their interaction with the surrounding medium, colloidal particles undergo random (Brownian) motion. They will diffuse from high to low colloid concentration, which means, from the aerosol cloud to the walls of the respiratory tract. The rate of this process is inversely proportional to the particle size and diffusion is therefore the dominant mechanism of deposition in airways for particles smaller than 0.5 μm, so called ultrafine particles (Gonda, 1988).

2.3.2.4 Interception

This mechanism occurs when part of the particle surface comes into contact with an airway wall (solid or liquid) even though the particle's center of gravity may lie in an

air streamline relatively distant from the wall (Agnew 1984). This mechanism is important for deposition of elongated particles, such as mineral fibers, in lower airways (Gonda, 1988).

2.3.3 Factors affecting drug deposition

Drug distribution and aerosol particle deposition in the lungs depends on several factors, some of them are explained in the following paragraphs.

2.3.3.1 Properties of the aerosol

Properties of the inhaled particles such as size, shape, diffusivity, density, viscosity, electrical charge and hygroscopicity, directly influence their deposition in the respiratory system (Martonen and Katz 1993). Aerosol size is probably the most important factor determining the deposition (Hickey 1992). It has been suggested that close attention to the droplet size of the aerosolized drug may be particularly important in the treatment of certain conditions, where penetration to the peripheral airways is particularly desirable, for instance the antibiotic treatment of cystic fibrosis (Newman and Clarke 1983; Newman, Woodman et al. 1988; Dennis 1994; McCallion and Patel 1996). The lung has a relative humidity of approximately 99.5 %. The addition or removal of water can significantly affect the particle size of a hygroscopic aerosol and thus deposition. Growth of hypertonic aerosols or shrinkage of hypotonic droplets should be avoided by administering isotonic aerosols (Phipps, Gonda et al. 1994).

2.3.3.2 Lung morphology

Physicochemical properties of the airways mucous, temperature, relative humidity, absorption, metabolism, and disposition are factors affecting deposition. Some changes in lung morphology are disease-induced and can therefore be predictable depending on the intensity of the disease. Bronchoconstriction, inflammatory lung diseases or repeated mucosal injury, by for instance allergens, viruses and pollutants can conduct to chronic structural changes to the airways. Such changes in tissue composition can in general be associated with lung dysfunction.

2.3.3.3 Breathing pattern

Breathing pattern parameters are such as tidal volume, frequency of inhalation/exhalation and flow. Slow inspiration of the aerosol reduces impaction in

the mouth and pharynx. Breath holding at the end of inspiration allows the particles that have moved beyond these regions to deposit by diffusion and sedimentation. The physical characteristics of the subject who inhales the aerosol (Newman 1984) and the breathing technique will directly affect lung deposition of drugs inhaled through devices such as nebulizers (Newman 2000).

2.3.4 Mechanisms of drug absorption in the lung

The most significant barrier to absorption of inhaled drugs is the epithelium of the lung (Patton 1996). This tissue is thick (50-60 μm) in the trachea, and extremely diminishes to 0.2 μm in the alveoli. Small molecules (100 to 1000 g/mol) with hydrophobic character are absorbed even more rapidly that those having some water solubility. Hydrophilic small molecules absorb within minutes to tens of minutes, while hydrophobic small molecules will absorb within seconds to a few minutes (Patton, Fishburn et al. 2004). As the drug in study in this work has a low molecular weight of 326 g/mol, a short review of the mechanism of absorption of small molecules in the lung will be given.

2.3.4.1 Transcellular diffusion

Lipid soluble compounds are rapidly absorbed because apparently they can be integrated into the lipid bilayer surrounding the cells. Compounds pass from the apical to basolateral side by travelling through the cellular membrane. There always can be molecule-specific interactions that delay or help to slow absorption, e.g. amphotericin B with endogenous sterols (Beyer, Schwartz et al. 1994) or due to charge-induced interactions taking place on the surface of cell membranes as the case of pentamidine (Monk and Benfield 1990). The encapsulation of small molecules (e.g. liposomes) can also result in a slower release (Pinto-Alphandary, Andremont et al. 2000).

2.3.4.2 Paracelullar diffusion

Hydrophilic compound most likely traverse the epithelium passing through aqueous pores in the intercellular tight junctions, in a mechanism that seems to be dependent of the molecular weight and ionisation grade of the molecule. Less ionised molecules will absorb faster as they have less interaction with the protein and lipids that line the pores.

2.3.4.3 Carrier mediated transport

Molecules following this process bind to specific carrier proteins on the cell surface (e.g. the high-affinity peptide transporter PEPT2) and use energy-driven exchange to drive uptake against a concentration gradient (Patton, Fishburn et al. 2004).

2.4 Current formulations and devices for oral inhalation of poorly water soluble drugs

2.4.1 Aqueous suspensions for nebulization

Currently several nebulizer formulations are marketed for the treatment of acute and severe diseases. Most of these treatments define a nebulizer system which is optimal to reach the site of deposition in the upper or lower airways and consequently achieve the therapeutic effect. Typical examples are antimicrobials like tobramycin (TOBI®, PathoGenesis Corp., Seattle, WA) used in the treatment of *P. aeruginosa* in cystic fibrosis patients; pentamidine isethionate (Nebupent®, American Pharmaceutical Partners, IL, USA) in the prophylaxis of *Pneumocystis* pneumonia in HIV patients. Nebulization is used also for the administration of bronchodilator drugs in acute and chronic severe exacerbations of asthma and chronic obstructive pulmonary disease (COPD) such as albuterol and ipratropium (DuoNeb® Dey, L.P., CA, USA). Also for the treatment of pulmonary hypertension, the drug iloprost is available as nebulizer formulation (Ventavis®, Schering, Berlin Germany).

All these products in the market are relatively unproblematic, as they all are water soluble and the nebulizer formulations are drug solutions. An aqueous dispersion for nebulization available in the market is the product Pulmicort Respules® (Astra Zeneca, DE, USA) a sterile suspension for inhalation via jet nebulizer and contains the active ingredient budesonide (micronized). A nebulizer budesonide nanosuspension conformed by budesonide nanocrystals (Sheffield Pharmaceuticals, Rochester, NY) was tested for efficiency against the formulation in the market (Pulmicort Respules®). Pharmacokinetic studies suggested a more rapid rate of delivery (C_{max}, t_{max}) of nanobudesonide with a similar extent of absorption as the marketed product. Nanobudesonide showed shorter nebulization time (Kraft, Steiger et al. 2004).

2.4.2 Suspensions for pressurized Metered Dose Inhalers (pMDI's)

Pressurized metred dose inhalers (pMDI's) are multidose systems, readily available in clinical use in which the drug is contained in a pressurized container and is expelled by a valve in a metered volume from a volatile propellant (Atkins, Barker et al. 1992).

In an attempt to reduce the destruction of the ozone layer and as a positive environmental contribution, the Montreal protocol reached the agreement of avoid the use and manufacture of chloro fluoro compounds (CFC's). This has forced the pharmaceutical industry to use substitutes like hydro fluoro alkanes (HFA's) such as HFA 134a. The no prompt substitution of CFC 11, used in the formulation of most of the pressurized Metered Dose Inhalers (pMDI's), made many companies to reformulate their products as a Dry Powder Inhaler (DPI) (e.g. asthma treatments). With the appearance of more HFA compounds, the formulation of pMDI's is again being developed. The table 2-1 list some of the colloidal products used in current treatments by means of a pMDI device.

Table 2-1: pMDI's marketed products based in dispersion formulations

Trade name	Active compound	Manufacturer	Therapeutic use
Ventolin®	Albuterol	GlaxoSmithKline	Bronchodilator
Azmacort®	Triamcinolone acetonide	Kos Pharmaceuticals Inc	Prophylactic therapy of asthma
Flovent® HFA	Fluricasone propionate	GlaxoSmithKline	Corticosteroid bronchodilator
Combivent®	Ipratropium bromide and albuterol sulphate	Boehringer Ingelheim Pharmaceuticals, Inc	Anticholinergic bronchodilator in patients with COPD
AEROBID® Inhaler System	Flunisolide	Forest Pharmaceuticals, Inc.	Corticosteroid anti-inflammatory, anti-allergic

New technologies are emerging as an attempt to deliver poorly water soluble drugs by means of pMDI. One of these is the PulmoSphere™ technology. It consists of a submicron fluorocarbon-in-water emulsion stabililised by a monolayer of long chain

phospholipid. The micronized drug is then dispersed in the continuous water phase and spray-dried. The fluorocarbon serves as a blowing agent, resisting droplet shrinkage and yielding hollow particles (Tarara, Hartman et al. 2004).

2.4.3 Powders and solid dispersions for use with Dry Powder Inhalers (DPI's)

Dry powder inhalers (DPI's) are devices in which the drug particles to be aerosolized must have the appropriate size distribution for deposition in the desired parts of the respiratory tract because these generators usually are not capable to break solid particles. Agglomerates of particles can be broken by a wire mesh or a propeller driven by the air flow, placed downstream towards the patient's mouth (Gonda 1988). Recently developed inhalers (such as Pulmonary Delivery System from the company Nektar) have a mechanism with ability of disaggregate efficiently inhalation powders. The table 2-2 show some of the DPI's existent in the market and their mechanism of action.

Drugs are micronized to achieve the optimal practical size that facilitates the drug deposition, as the case of corticosteroids used in asthma treatments such as budesonide Clickhaler® (Merck dura), budesonide Sandoz® Easyhaler (Sandoz) or a combination of budesonide and formoterol Symbicort® Turbohaler® (Astra Zeneca). Others, like insulin, are first transformed to spherical particles of an amorphous powder produced by spray-drying. The product Exubera® (Pfizer Pharma) is now in the market delivered with a Pulmonary Delivery System, a sonic discharge of air through the novel TransJector reproducibly extracts, deagglomerates, and disperses the inhalation powder (White, Bennett et al. 2005).

Table 2-2: Examples of DPI's currently in the market and their mechanism of action

DPI Model	Manufacturer	Mechanism of action
Rotahaler®	GlaxoSmithKline	Passive mechanism activated by the patient's breath. The drug is dispensed by twisting action on the base of the unit
Diskhaler® Diskus®	GlaxoSmithKline	Each dose of medication is contained in a blister. The device punctures each blister and the drug can be inhaled by a passive mechanism activated by the patient's breath
Clickhaler®	Merck dura	Passive mechanism activated by the patient's breath
Gyrohaler®	Vectura	Passive mechanism activated by the patient's breath. Drug is in foil-sealed system.
Turbuhaler®	Astra Zeneca	Passive mechanism activated by the patient's breath. The drug is dispensed by twisting action on the base of the unit
Spiros	Dura Pharmaceuticals Inc.	Active mechanism. Battery powered turbine
MAGhaler	Mundipharma, Frankfurt Germany	Active mechanism. Mechanical (spring driven) scraper
Aspirair®	Vectura	Active mechanism.
Powderhale®	Vectura	Active mechanism.
Pulmonary Delivery System	Nektar	Active mechanism with compressed air released by means of a "TransJector".

2.5 Nebulization

By definition, an aerosol is a colloidal dispersion of a liquid or a solid in a gas. The word nebulization comes from the Latin term for "mist", which is "nebula" (Lloyd 1996).

2.5.1 Basic principles of atomisation theory

2.5.1.1 Generation of particles with ultrasonic nebulization

The generation of clouds of droplets by means of ultrasonic waves was first reported in 1927 (Wood and Loomis 1927; Barreras, Amaveda et al. 2002). Two different mechanisms explain ultrasonic atomization, capillary waves and cavitation. The Kelvin equation for capillary waves is described as:

$$\lambda = \left(\frac{2\pi\sigma}{\rho f^2}\right)^{1/3}$$

where:

λ = Wavelength of surface waves at the air/liquid interface

σ = Surface tension coefficient

ρ = Liquid density

f = Frequency of the surface waves

After further investigations, Rayleigh modified Kelvin's equation and derived the following expression:

$$\lambda = \left(\frac{8\pi\sigma}{\rho F^2}\right)^{1/3}$$

where F is the forcing sound frequency or frequency of the acustic signal. These theoretical basis were already established when in 1927 the possibility of atomizing liquid by exciting them with ultrasonic waves was described. Years later, in 1962, Lang published the expression relating the wavelength to droplet size D through an empirical constant, which he experimentally reported to be 0.34 (Barreras, Amaveda et al. 2002).

$$D = 0.34\left(\frac{8\pi\sigma}{\rho F^2}\right)^{1/3}$$

Based in this principle, new devices in the market operate at higher frequencies, aiming to reduce the size of the droplets, while formulations to be nebulized under this principle should have higher densities and lower surface tension values in order to achieve lower droplet diameters. It needs to be considered that experiments and

equations described above have been described for aqueous droplets of solutions and not with colloidal dispersions. The use of nanosuspensions in nebulizers based in this mechanism seems to be an attractive approach, as the surface tension of the aqueous phase is strongly reduced due to the presence of surfactants, necessary for the stabilization of the drug nanocrystals.

2.5.1.2 Generation of particles with vibrating orifice ultrasonic nebulization

This method is based in the disintegration of a jet of liquid issuing from an orifice that is driven by a periodic vibration of the appropriate frequency and amplitude. Uniform droplets can be produced by vibrating the liquid, the container holding the liquid or the orifice, being the droplet size primarily controlled by the size of the orifice (mesh). The particle diameter, d, can be calculated from the ratio of the liquid federate Q, to the frequency of vibration, f, applied if the particle density ρ_p, is the same as the bulk material used:

$$d = \left(\frac{cQ}{\pi f \rho_p} \right)^{1/3}$$

where c is the mass concentration of the solute per unit volume of the solution. In the case of a pure liquid, c has the value of 1 (Leong 1992).

2.5.1.3 Generation of particles with jet nebulization

For the case of jet nebulizers, the droplet size produced will be not only dependent on the design of the nebulizer itself (nozzle diameter, speed of the compressed air flow), but also from the intrinsic characteristics of the liquid or suspension to be nebulized such as viscosity and surface tension. It has been suggested that droplet size was inversely proportional to the relative velocity between the air and the liquid and proportional to the liquid's surface tension (for low viscosity liquids). In 1981, Mercer derived and equation relating the primary droplet diameter (d) with the diameter of the venturi nozzle (D) (McCallion, Taylor et al. 1995):

$$d = 0.64\, D \left[1 + 0.011 \left(G_l / G_g \right)^2 \right] \left[2\gamma / \left(9 \rho \upsilon^2 D \right) \right]^{0.45}$$

where:

D = Diameter of liquid inlet orifice

G_l = mass flow-rate of liquid

G_g = mass flow-rate of gas

γ = surface tension of liquid

ρ= density of the gas

υ = velocity of the air

The liquid mass flow-rate can be affected by the viscosity η of the liquid, which will resist the droplets formation and can increase the size of the droplet (see equation). Reduced surface tension should in any case produce smaller droplets (McCallion, Taylor et al. 1995; McCallion and Patel 1996; McCallion, Taylor et al. 1996). For jet nebulizers, the efficiency of the device to produce aerosol droplets defined here as atomization rate, A^2, can be defined by the following properties of the liquid phase:

$$A^2 = \frac{\pi\rho}{\eta\gamma}$$

where:

ρ= liquid vapour pressure

η = viscosity

γ = surface tension of liquid

It can therefore be expected that formulations including a surfactant with low viscosities, will not only reduce the size of the droplets produced by a jet nebulizer device, but also will increase the output of the liquid formulation i. e. the drug output.

2.5.2 Types of nebulizers

2.5.2.1 Jet nebulizers

From all the jet nebulizers in the market, probably the most popular systems are those of the company Pari GmbH, e.g. Pari LC Plus® and Pari LC Star®. These systems belong to the so called jet enhanced nebulizers. They have a valve on top of the sample reservoir that will open during inspiration, letting more air pass and draw a higher quantity of aerosol particles into the inspired air stream. The same valve will close during exhalation, reducing the flow inside of the nebulizer chamber and with it the loss of aerosol during this phase (See Figure 2-2). Conventional jet nebulizers will aerosolize continuously, resulting in higher losses of dose and shorter nebulization times (Ledermüller, Stangl et al. 2003).

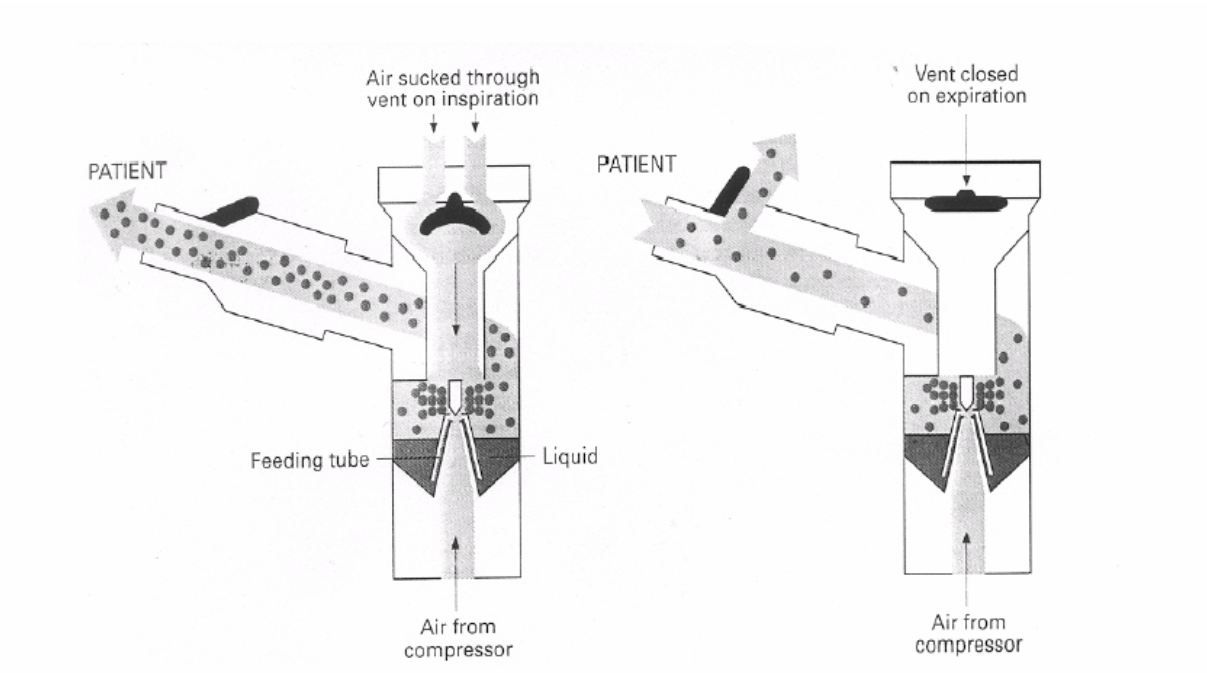

Figure 2-2: Example of an enhanced jet nebulizer showing the aerosol flow during inspiration and expiration phases (O' Callaghan and Barry 1997).

In these nebulizers, the driving gas (coming usually from a compressor device) passes through a very narrow hole, known as a venturi. At the ventury, the pressure falls and the gas velocity increases greatly producing a cone shaped front. This passes at high velocity over the end of a narrow liquid feed tube or concentric feeding system creating a negative pressure at this point. As a result of this fall in pressure, liquid is sucked up by the Bernoulli effect and is drawn into fine ligaments. The ligaments then, collapse into droplets under the influence of surface tension. The primary generation (atomization) typically produces droplets 15-500 μm in diameter. Coarse droplets impact on baffles while smaller droplets may be inhaled or may land on internal walls returning to the reservoir for renebulization (Nerbrink, Dahlback et al. 1994; O' Callaghan and Barry 1997). Although all jet nebulizer have basically the same principle, different jet nebulizers have different output characteristics determined by the design of air jet and capillary tube orifices, their geometric relationship with each other and the air jet and capillary tube orifices.

2.5.2.2 Ultrasonic nebulizers

In ultrasonic nebulizers, the energy for atomizing liquids comes from a piezoelectric crystal vibrating at high frequency. The origin of the aerosol droplets can be direct or indirect depending on the technology as is explained in the following paragraphs.

- Ultrasonic nebulizers

The piezoelectric crystal is in direct contact with the formulation such as Multisonic® (Schill GmbH & Co. KG, Probstzella, Germany). This ultrasonic nebulizer uses a rapidly vibrating piezoelectric crystal to produce aerosol particles. Ultrasonic vibrations from the crystal are transmitted to the surface of the drug solution were standing waves are formed. Droplets break free from the crests of these waves and are released as aerosol. The size of droplets produced is inversely proportional to the power of the acoustic frequency. Like jet nebulizers, baffles within the nebulizer remove large droplets and much of the aerosol produced impacts on these, falling back into the drug reservoir.

- Passive vibrating mesh ultrasonic nebulizers

An example of this category of nebulizers is the Omron Micro Air NE-U22 (Omron Health Care Ltd., Milton Keynes, UK), which works at 180 kHz frequency or the Omron U1 (65 kHz). It is a small battery-operated nebulizer in which vibrations of the piezoelectric crystal in an ultrasonic horn are used to force drug solution through a mesh of hundreds or thousands of micron-sized holes, creating an aerosol.

- Active vibrating mesh ultrasonic nebulizers (electronic mesh systems)

In these devices, the mesh itself is vibrated directly by a piezoelectric crystal. To this mechanism belong AeroNeb®Pro and AeroNeb®Go (Nektar Therapeutics), e-Flow™ (Pari GmbH) and TouchSpray™ (ODEM Ltd.). They use a perforate membrane, which vibrates at ultrasonic frequencies, in contact with the reservoir fluid, to generate the aerosol cloud. The vibration action draws jets of fluid though the holes in the membrane (micro-pumping action), breaking the jets into drug cloud. The size of the droplets is controlled by the shape/size of the holes as well as the surface chemistry and composition of the drug solution.

2.5.2.3 Electrical nebulizers

This technology uses an electrohydrodynamic (EHD) aerosolization mechanism for dispersing the liquid formulation. One example of this category is the device Mystic™ (Ventaira Pharmaceuticals Inc.). It uses strong electric field to break liquid into a spray of nearly monodispersed, charged particles. The negative charge tends to remain on the surface of the liquid such that, as the liquid exits the nozzle, the repelling force of the surface charge balances against the surface tension of the liquid, forming a cone. The electrical force exerted on the liquid surface overcomes

the surface tension at the tip of the cone, generating a thin jet of liquid. This jet breaks into droplets of more or less uniform size, which collectively form a cloud.

2.5.2.4 New "mixed" nebulizers

New nebulizers such as Respimat®Soft Mist™, a system marketed in 2003 by the company Boehringer Ingelheim GmbH, is a mixed form of nebulizer and pMDI. The mechanism is propellant free and delivers a metered dosage of medication as a fine mist. The medication is stored in a collapsible plastic bag, in a sealed plastic container inside of the cartridge. When the dose-release button is pressed, the energy released from the spring forces the solution through a "uniblock", releasing a slow-moving aerosol. The uniblock is the extremely fine nozzle system producing two fine jets of liquid, when the medication solution is forced through it. The two jets of liquid converge at an optimised angle, and their impaction generates the aerosol. The use of this kind of systems has not been published with suspensions.

2.5.3 Factors affecting nebulizer performance

In the administration of drugs using nebulization devices, the dose of drug delivered to the lung depends on a number of factors including the method of aerosol generation and delivery, the volume of solution in the nebulizer and the method of inhalation (Newman 1985).

2.5.3.1 Humidity and temperature of ambient air

The temperature and humidity of the ambient air plays a role in influencing aerosol output and particle size. At higher ambient temperatures, particle size decreases due to increased evaporation of the diluent. The physical-chemical effects that influence the size distribution of droplets are known. Coagulation, evaporation and condensation of solvent water depend on the relative humidity of the surrounding air. In addition, the vapor pressure at the curved surface of small droplets is elevated favoring evaporation (Kelvin effect). There are about 0.5 - 2 sec between production and inhalation, depending on which nebulizer and inhalation equipment is used. During this time, the original size distribution can change drastically (Ferron and Soderholm 1990; Ferron, Roth et al. 1997). It is suggested that when using nebulizers, two droplet size distributions have to be distinguished: the original distribution at the time of nebulization and the size distribution one or more seconds

after nebulization. The latter is important for medical applications (Porstendörfer, Gebhart et al. 1977).

2.5.3.2 Baffles

The presence of baffles into the design of the nebulizer will reduce the particle size of the aerosol emitted. Baffles in nebulizers ensure that the large particles impact the baffles and drop back into the chamber (Kendrick, Smith et al. 1997).

2.5.3.3 Characteristics of the solution

Determinants of droplet size produced by nebulizers include the properties of the formulation such as density, viscosity, surface tension (Dolovich 1989; McCallion, Taylor et al. 1994; Phipps and Gonda 1994; McCallion, Taylor et al. 1995; McCallion and Patel 1996; Lintz, Walther et al. 2002)

2.5.3.4 Solution volume or fill volume

Fill volume means the volume of drug solution initially put into the nebulizer chamber. It must exceed the residual volume by a sufficient amount to provide therapeutic benefit to the patient. It is suggested that it should be at least twice the residual volume (Kendrick, Smith et al. 1997). With low fill volumes, the dead volume will represent a significant fraction of the nebulizer contents (Dolovich 1989).

2.5.3.5 Operating time

The time taken to deliver the drug is important for patient's compliance. The optimum time for nebulization is 5-10 min. Patients will generally not accept long delivery times, especially if the treatment is required several times per day. The end point of nebulization needs to be defined. There is some evidence that nebulising "to dryness" is confusing for patients. Jet systems nebulise continuously until the fill volume approaches the residual volume and "sputtering" occurs. (Kendrick, Smith et al. 1997).

2.5.3.6 Air pressure to the jet

The flow of the driving gas and the performance characteristics of the compressor has an important role in the nebulization process using jet nebulizers (Kendrick, Smith et al. 1997). Flow was shown to have a little effect on nebulizer output, but it did affect nebulization time and particle size. A higher flow resulted in a shorter nebulization time, which could be used clinically to offset the effect of a larger diluent

volume on nebulization time. Increasing the flow increased the proportion of particles in the respirable range of 1-5 μm. The effect of flow on particle size was greater between 6 and 8 L/min than between 8 to 10 L/min. Increasing flow increases the number of particles in the respirable range but also increases the amount of waste during the expiratory phase so that a respirable mass remains relatively constant. This is supported by previous studies (Hess, Fisher et al. 1996). Humidity and temperature of the driving gas also have been shown to have an influence.

2.5.3.7 Dead volume

The dead volume is typically in the range of 1-3 mL (Hess 2000). The fill volume does not appear to influence the particle size distribution (Kendrick, Smith et al. 1995). The residual volume of the modern, small volume, nebulizer chambers is less than 1.0 mL and for these, giving flexibility for using a fill volume of 2-2.5 mL of drug formulation, when assuming a minimum fill volume of approximately the double of the residual volume (Kendrick, Smith et al. 1997).

2.5.4 Problems with conventional dispersions during nebulization of lipophilic drugs

Drug suspensions are inherently more complicated to nebulize as they are a mass of suspended particles which may or may not be present within the droplets which is clinically important, whereas with solutions, it is assumed that the entire drug is homogeneously dispersed throughout all droplets. For example, conventional ultrasonic nebulizers cannot be used to administer suspensions such as nebulized budesonide (Boe, Dennis et al. 2001).

It has been previously shown that conventional ultrasonic nebulizers generally perform poorly when filled with suspension formulations of non-water soluble drugs such as inhaled corticosteroids (Nikander, Turpeinen et al. 1999). Another study with latex particles in suspension has shown that ultrasonic nebuliser was less efficient than the jet devices and that it was unable to atomise the 1.16 μm sphere suspensions (McCallion, Taylor et al. 1996). An in vitro study (Yoshiyama, Yazaki et al. 2002) showed that the MicroAir Ne-U22 (Omron Healthcare Europe B.V) can nebulise up to 70% of a commercially available suspension formulation (budesonide suspension, Pulmicort repsules, Astra Zeneca). This shows the effort made by nebulizer manufacturers to produce more efficient nebulizers that can nebulize suspension formulations. Besides the design of new technologies, the development

of nebulizer formulations with solids in an appropriate size, as the nanosuspensions proposed in this work, can be an alternative to use nebulization systems already existent in the market, which are capable to produce droplets not only in the range of upper airways deposition (3-5 µm) but under 3 µm, to local deposition in the alveoli.

2.6 Pneumocystis pneumonia

2.6.1 Aetiology and epidemiology

In 1909 Chagas first isolated *Pneumocystis* from rats infected with *Trypanosoma cruzi (Chagas 1909).* In 1910, Carini confirms their existence as a morphologic form of the *Trypanosome* life cycle (Carini 1910). Both classified *P. carinii* in the protozoa group. Many years later, in 1988, *P. carinii* was accepted in the scientific community as part of the fungi kingdom. srRNA analysis demonstrated that *P. carinii* has similarities to Saccharomyces and Neurospora (Edman, Kovacs et al. 1988; Stringer, Stringer et al. 1989). Although its phylogenetic classification, antimicotic drugs have no effect against *P. carinii* and its cultivation in typical fungi culture media was until now not successful. In further studies to support its classification, Gigliotti, *et al*. in 1993 conducted transmission tests between different host species and found host-specific strains of *P. carinii* with different molecular- and antigen structure (Gigliotti, Harmsen et al. 1993). Due to significant genomic and phenotypic differences between *Pneumocystis* isolated from human and animal hosts, the nomenclature has undergone major revisions in the past years. Although the taxonomic position of *P. carinii* is still uncertain, it is conventionally accepted that the common term "*Pneumocystis carinii*" now either describes a group of *forma specialis* (Anonymous 1994; Durand Jolie, Aliouat et al. 2002) referring to their selective infectivity for certain hosts, or, *sensu strictu*, to one of the two species found only in rats. Following the latter nomenclature and more recent studies, laboratory mice contain a distinct species of *Pneumocystis*, *Pneumocystis murina* (formerly *P. carinii* f. sp. *muris*) (Keely, Fischer et al. 2004). The pathogen infecting humans has been proposed to be renamed as *Pneumocystis jiroveci* (formerly *P. carinii* f. sp. *hominis*) in honour of the Czech parasitologist Otto Jirovec, who is credit with describing the pathogen in humans (Frenkel 1999; Stringer, Beard et al. 2002). In the 40's and 50's he reported the pathogen in undernourished and premature infants presenting interstitial plasmacellular pneumonia in Europe (Jirovec 1952; Vanek, Jirovec et al. 1953). The term *Pneumocystis* pneumonia is the actual description of the acronym PcP (Agarwal

2005). PcP is an airborne, opportunistic infection caused by an ubiquitous, unicellular fungus (Stringer 1993).

Several seroepidemiological studies have shown that the vast majority of the human population becomes seropositive to *Pneumocystis* by the age of 4 years, attesting to the suspected ubiquitous distribution of the organism (Meuwissen, Tauber et al. 1977; Cushion 1994). Normally, an infection remains sub-clinical and is spontaneously cleared by the immune systems. Effective immunity against pathogens is known to be mediated by cellular immunity, with the complex interaction among T-lymphocyte populations, macrophages, cytokines, and the *Pneumocystis* (Johnson and Phil 1977; Pesanti 1994). The infection is progressive, leading to interstitial pneumonia, carnification of the interstitial tissue, damage to the alveolar lining, and accumulation of exudate within the alveoli (Höffken, Bäthge et al. 1999). Enhanced susceptibility for PcP is typically found among transplant recipients receiving immunosuppressive, and cancer patients receiving cytostatic therapy or those with congenital immune diseases (Hughes, Feldman et al. 1975; Saulsbury, Bernstein et al. 1979; Wazir and Ansari 2004). For HIV-infected individuals, PcP is one of the AIDS-defining complications (Wakefield 2002). If left untreated and the immune deficit persists, PcP is fatal. Therefore, high-risk patients generally receive PcP-prophylaxis, as part of the highly active antiretroviral therapy (HAART) when their $CD4^+$ T lymphocyte count is below 200 cells/µl (Schneider, Borleffs et al. 1999). A study of solid organ transplant recipients suggested that lung transplant recipients were at continued risk of developing PcP and that prophylaxis may need to be continued indefinitely in this group (Gordon, LaRosa et al. 1999). Patients with pneumonia resistant to treatment are a common problem in chest hospitals. PcP should always be considered as an opportunistic pathogen in case of potential, especially T cell-related immunodeficiency, even if AIDS is not obvious. Cases of PcP (n = 1921) without associated AIDS in a chest hospital has been also recently reported (Blum, Roth et al. 2006).

Progress towards understanding their life cycle, metabolism and basic biology has been hindered owing to the inability to continuously propagate any species outside the lung (Cushion 2004). The various species of *Pneumocystis* are extracellular organisms in the lung alveoli. The intrapulmonary life cycle of the pathogen involves asexual reproduction of haploid trophic forms by cell division, and the sexual formation of reproductive cysts (sporogenesis), involving meiosis and mitosis to form

eight intracystic bodies (spores) within the thickened cyst wall (spore case) (Ruffolo 1994). In routine microscopy, two prevalent forms are normally found: small, haploid trophozoits (1-4 μm) and bigger multinuclear (until eight nucleuses) cysts (5-8 μm). The Figure 2-3 shows the life cycle explanation reported by Cushion, *et. al.* 1988. Trophic forms tend to adhere to type I pneumocytes in the lung alveoli, and this property is an important aspect of the pathogenesis of *Pneumocystis* infection. The adhesion between the pathogen forms to organise clumps and the attachment of *Pneumocystis* trophic forms to type I pneumocytes likely are mediated by surface polysaccharides (Ruffolo 1994).

After inhalation or aspiration of the infectious form, *Pneumocystis* establishes a parasitic life cycle. At this stage host effector mechanisms that prevent the initial attachment (e.g. antibody to a key attachment protein) or kill the infectious particle (e.g. alveolar macrophages) could intervene. In the nonimmune host, attachment to alveolar Type I epithelial cell membranes firmly occurs, in what appears to be an essential aspect of the intra-alveolar life cycle of the pathogen. Although *Pneumocystis* is apposed to alveolar Type I cells, it is covered by a layer of alveolar lining fluid, consisting of surfactant and other substances. It may be relatively inaccessible to phagocytes and immunoglobulins at this stage, but cytokine stimulation of type I cells may inhibit the growth of the pathogen. It is probably at this stage, when the cellular immunity may be most effective in defenses against it. Subsequently, Pneumocystis multiplies and breaks free from the now dead or dying epithelial cell. Further multiplication within the alveolar airspaces then occurs, eventually resulting in the characteristic cysts containing eight daughter cells (Pesanti 1994).

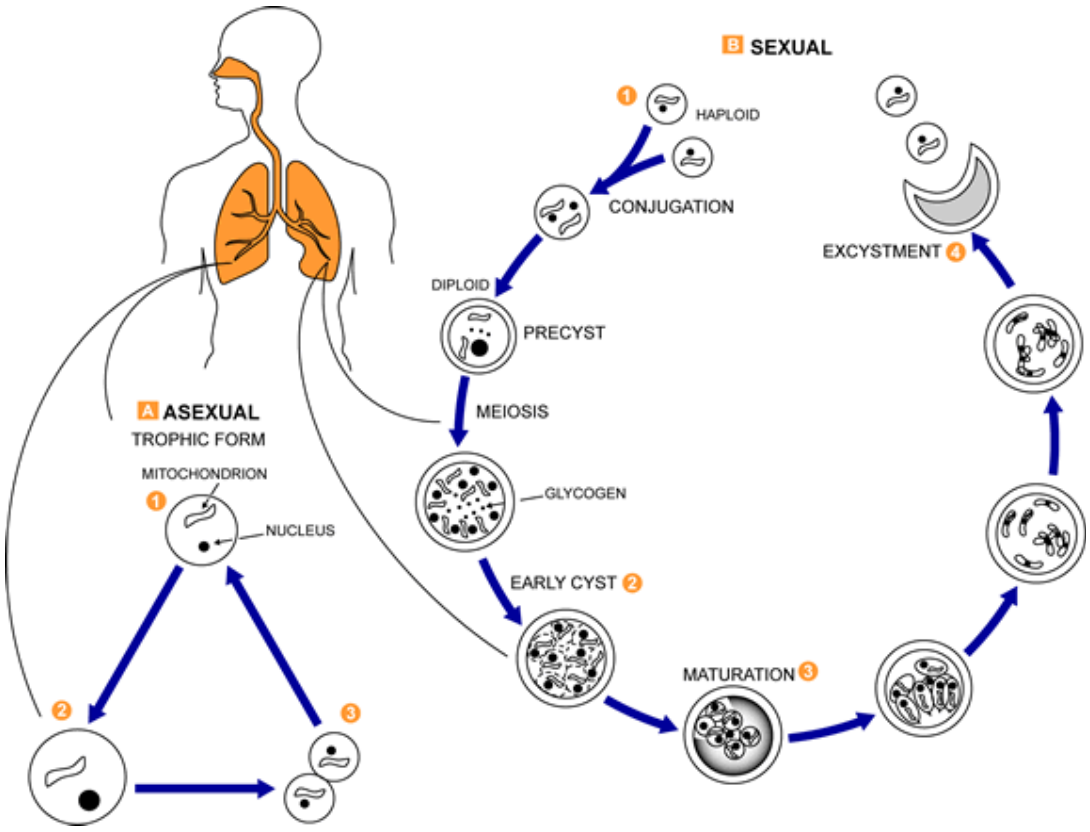

Figure 2-3: Pneumocystis life cycle. Drawing by Dr. J.J. Ruffolo (taken from (Cushion 1998)). Asexual phase: trophic forms 1 replicate by mitosis 2 to 3. Sexual phase: haploid trophic forms conjugate 1 and produce a zygote or sporocyte (early cyst) 2. The zygote undergoes meiosis and subsequent mitosis to produce eight haploid nuclei (late phase cyst) 3. Spores exhibit different shapes (such as, spherical and elongated forms). It is postulated that elongation of the spores precedes release from the spore case. It is believed that the release occurs through a rent in the cell wall. After release, the empty spore case usually collapses, but retains some residual cytoplasm 4. A trophic stage, where the organisms probably multiply by binary fission is also recognized to exist. (Centers for Disease Control & Prevention, National Center for Infectious Diseases et al. 2005)

2.6.2 Current drugs used for prophylaxis and therapy

The drug initially prescribed for the prophylaxis and treatment of PcP is the combination of sulfamethoxazole and trimethoprim (SXT), which in numerous comparative trials has been demonstrated to have efficacy very similar or superior to that of other agents (Klein, Duncanson et al. 1992; Hughes, Leoung et al. 1993; Bozzette, Finkelstein et al. 1995; Toma, Thorne et al. 1998). Despite its broad spectrum of efficacy, many patients may experience treatment-limiting side effects such as rash, renal dysfunction, hepatitis and bone marrow suppression, showing the necessity of alternative drugs (Hughes, Leoung et al. 1993; Hughes, LaFon et al. 1995). In the case of mild-to-moderate *Pneumocystis* pneumonia, alternatively to SXT as the first-line prophylactic and therapeutic agent, intravenous pentamidine, trimetrexate, dapsone, primaquine-clindamycin and atovaquone (ATQ) may be used when standard treatment is intolerable or ineffective (Hughes, Leoung et al. 1993; El-Sadr, Murphy et al. 1998; Chan, Montaner et al. 1999; U.S. Public Health Service and Infectious Diseases Society of America Prevention of Opportunistic Infections Working Group 2002).

Nebulizer therapy of PcP has been investigated in an attempt to deposit high concentrations of pentamidine in the alveoli (Simonds, Newman et al. 1990). In this study, the alveolar deposition was greatest and the side effects least with the nebulizer that produced the smallest droplet size profile (Respigard II), whereas large airway-related side effects were most prominent and alveolar deposition lowest with the nebulizer producing the largest droplet size (Kayser and Kiderlen 2003). Today, aerosolized pentamidine is a second line therapy to be used in the prophylaxis of *Pneumocystis* pneumonia in patients who also suffer from AIDS. Atovaquone has shown similar efficacy than aerosolized pentamidine (Chan, Montaner et al. 1999) in the prophylaxis of PcP. For this work ATQ was particularly relevant due to his use against PcP, due to structural similarities with the molecule buparvaquone.