CHAPTER 8

SUMMARY AND CONCLUSION

8.1 SUMMARY

Localized delivery of drugs to the respiratory tract has become an increasingly important and effective therapeutic method for treating a variety of pulmonary disorders, including asthma, bronchitis, and cystic fibrosis (CF). Patients with CF and chronic pulmonary infection with *Pseudomonas aeruginosa (Psa)* have frequent acute exacerbations of respiratory infection characterized by increased sputum production and tenacity; increased sputum purulence; increased frequency and severity of cough; malaise; deterioration of pulmonary function; weight loss; fever; and leukocytosis. Many patients are given long-term treatment with inhaled and/or systemic antibiotics in the hope of maintaining their physical well-being, quality of life, weight, and lung function, as well as to decrease the number of disease exacerbations and hospital admissions.

Antibiotic therapy has become a mainstay of treatment for patients with CF or non-CF bronchiectasis who are infected with *Psa*, and has been credited with the prolonged survival of CF patients. Patients with CF and chronic pulmonary infection with *Psa* requires much larger doses of antibiotics to the lungs than in the case of bronchodilators or steroids. Delivering large dose of medication to the lung has been a challenging task for scientists and manufacturers. Traditionally, aerosolized antibiotics have been administered by means of small-volume nebulizers, like preservative-free concentrated solution of tobramycin sulfate. Drug administration by nebulizers is cumbersome, time consuming, and expensive. For treating reversible airflow obstruction with bronchodilators and steroids, nebulizers have been increasingly replaced by dry powder inhalers (DPIs) and are proven to be efficient in delivering such high doses, producing no apparent bronchial irritation or systemic absorption of the drug . Conventional methods for producing DPI formulations containing high therapeutic payload have been prepared as micronized drug in carrier-based system and as a self-agglomerated aerosol formulation. Carrier-based systems generally contains large carrier particles comprising lactose to prevent the fine drug particles from aggregating and allow for at least some of the drug particles to loosely bind to the lactose surface and de-aggregate upon inhalation. However, substantial amounts of the drug fail to de-aggregate from the large lactose particles and are deposited in the throat. As such, these carrier systems are relatively inefficient with respect to the fine particle fraction provided per actuation.

Therapeutic dry powders traditionally comprise air born suspensions of particles with particle mass density (ρ) of ~1±0.5 g/cm^3 and mean geometric diameters (d) of < 5 μm to avoid excessive deposition in the DPI and oropharyngeal cavity. Fine powders (< 5 μm) generate fine aerosols, but particle adhesion reduces delivery efficiency and leads to flow rate dependant lung deposition. Hence, it would be advantageous to develop powder formulations with improved dispersibility. An attractive strategy for preparation of inhalation particles with improved aerosolization efficiency is by diminishing aerosol particle mass density and increasing particle size. Particle traits need to be engineered to produce aerodynamically light, ($\rho < \sim 0.4$ g / cm^3) and relatively large size (d > 5 μm), so that aerodynamic diameter fits into the window of 1-5 μm and can be successfully inspired into the lungs. Large particles aggregate less and deaggregate more easily under shear forces than fine (< 5 μm) particles. As a consequence of their large size and low mass density, these particles can aerosolize from a DPI more efficiently, resulting in higher respirable fractions of inhaled therapeutics. The large particle's high efficiency can be attributed to their smaller surface to volume ratio. Large particles aggregate less than small particles, all other considerations being equal. Thus while both have identical aerodynamic diameters, the large particles tend to exit the DPI more generally as single particles. The smaller particles aggregate more, leading to their deposition by gravity and inertia before reaching the respirable stage.

The human lungs can remove or rapidly degrade hydrolytically cleavable deposited aerosols over periods ranging from minutes to hours. In the upper airways, ciliated epithelia contribute to the "mucociliary escalator" by which particles are swept from the airways toward the mouth. In the deep lungs, alveolar macrophages are capable of phagocytosing particles soon after their deposition. As the diameter of particles exceeds 2 to 3 μm, very large particles deposited in the pulmonary region may escape clearance by alveolar macrophages and therefore, permit drug release for longer period of time and more efficiently. However increasing the particle size also minimizes the probability of particles (possessing standard mass density) entering the airways and acini due to excessive deposition in the oropharyngeal or nasal regions. An effective dry-powder inhalation therapy for both short and long term release of therapeutics, either for local or systemic delivery, requires a powder that displays

minimum aggregation, as well as a means of avoiding or suspending the lung's natural clearance mechanisms until drugs have been effectively delivered.

Aminoglycosides are considered among the most useful classes of antibiotics for treating *Psa* infections. The major drawback of aminoglycosides is the need for their relatively high-dose intravenous administration, which carries the potential for systemic toxicity. Consequently, when given intravenously in maximum safe doses, only relatively low sputum aminoglycoside concentrations are achievable. tobramycin sulfate (TB) and amikacin sulfate (AMK) were selected for the study, because of their relatively low systemic toxicity and minimum inhibitory concentrations. TB is one of the aminoglycosides with the lowest systemic and toxicity, and early empiric studies revealed that aerosol administration of the intravenous formulation to a variety of patients appeared to be well tolerated and it was more active then gentamycin against *Psa.* Efficacy of TB aerosol therapy has been clinically proven and improvement was observed by a substantial reduction in the numbers of *Psa* in the sputum and a decrease in the peripheral blood neutrophil count. AMK, a broad spectrum aminoglycoside effective against *Psa* at lower minimum inhibitory concentrations (MIC_{90}) of 2 μg/ml and retained their activity against gentamycin and TB resistant microorganisms, because of its unique resistance to the aminoglycoside-inactivating enzymes. Low sputum level of intravenously administered aminoglycosides and their associated systemic toxicities can be circumvented by direct delivery of effective amount of these antibiotics to the lower airways.

It was hypothesized that preparation of aerodynamically light and large particles (ALLP) of TB and AMK will result into improvement in flowability and fine particle fraction, which will lead to improved lung deposition (lower airways) by reducing deposits in the extrathoracic and tracheobronchial airways. It is further hypothesized that, lung antibiotic concentration will be maintained over sustained period; because of the role of large particles in escaping phagocytosis. The present approach seems to be ideally apposite for inhaled therapies used in the treatment of diseases involving infection of the airway (CF, non CF-bronchiectasis). Hence, this investigation was focused on the pharmaceutical development of ALLP of selected antibiotics, optimization of formulation components for maximizing aerosol performance and evaluation of the selected formulations in animals.

The drug content and the excipients of ALLP were analyzed by the reported analytical method with suitable modification whenever necessary to meet the requirement of this investigation. The method was standardized for estimation of drugs (TB and AMK) under study, hydrogenated soyaphosphatidylcholine (HSPC) and 6-coumarin contents. The ability of phospholipids to form a red colored complex with ferrothiocyanate in organic solutions was used to estimate HSPC. The method was found to obey beer/s law between 10 – 100 μg/ml concentrations of HSPC in chloroform. 6-coumarin was used as fluorescent marker in ALLP. Estimation of 6-coumarin in the formulation is a rapid and sensitive method for characterization of in-vitro aerosol performance. It emits fluorescence at excitation wavelength of 458 nm and emission wavelength of 545nm. Linearity of the method was observed from 100-1000 ng/ml.

TB and AMK has poor absorbance in UV and visible region, hence these were derivatized with absorbance enhancing agent 2, 4-dinitrofluorobenzene 2, 4, 6 - trinitrobenzene sulphonic acid respectively. Extraction of TB/AMK into aqueous media followed by derivatization shown absorption maxima at 365nm (TB) and 340nm (AMK) at retention time of 18 min (TB) and 12 min (AMK). Calibration curve for TB and AMK was linear over the range from 1 to 400μg/ml. Selectivity of method was estimated by analyzing the placebo formulations/ blank biological samples treated in similar manner to that test samples and injected. There was no interfering peak appeared in the chromatograms of real samples. Stability of TB/AMK-complex was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature. Estimation of TB/AMK from lung homogenate was carried out by extraction of drug followed by derivatization. Linearity was observed over the range from 1 to 200μg/ml for both TB and AMK. Methanol was selected as the optimal extracting solvent which enabled recovery up to at least 90%.

ALLP of AMK and TB was prepared by both spray drying (SD) and freeze drying (FD) process with varied proportion of drug (30-50% w/w) in the formulation. Spray and freeze drying parameters were previously optimized and the same operational parameters were used to prepare ALLP. SD process parameters, such as feed rate and pressure of the compressed air were found to have insignificant influence on bulk properties of ALLP, such as geometric particle diameters and bulk tap density. However, increases in the inlet temperature increased bulk tap density and decreased aerosol performance ($P > 0.05$). Hence, process parameters were optimized and kept

unaltered in subsequent experiments. Powder produced with superior aerosol performance was optimized statistically to establish the quantitative aspects of the effects and relationships among various formulation components of high therapeutics payload ALLP. A 3-factor, 5-level central composite design was used for the optimization of ALLP produced by SD. This design was suitable for exploring quadratic response surfaces and constructing second-order polynomial models. The complete design consisted of 16 experimental points that included eight vertex points, six axial points and two replications at the centre point. Each batch of ALLP was prepared three times and was evaluated for particle size, polydispersity, tap density and moisture content.

Marginal differences were observed in traits such as tap density and moisture content of ALLP containing 30-50%w/w of drug. Further increase in drug proportion resulted increase in density of ALLP. Both the spray and freeze drying techniques were found to be reproducible with high therapeutic payload ALLP. Significant difference in particle size (VMD) and polydispersity observed between SD and FD-ALLP. SD-ALLP of both TB and AMK were consistently drier then FD-ALLP. However, on subsequent vacuum drying, both ALLP reached same level of moisture content. DSC thermograms for FD and SD-ALLP containing TB revealed an endothermic peak between 274°C to 278°C, corresponding to the melting endotherm of pure TB at 272.17°C. The molar heat of fusion value for SD-ALLP (ΔH 199.49mJ) is higher then pure TB (ΔH 8.87 mJ), this could be due to crystallization of TB during the DSC run in molten formulation excipients (HSPC/poloxamer 188/L-leucine). Similarly a single endothermic peak was observed around 264 to 269°C in both FD and SD AMK –ALL samples, which corresponded with the melting endotherm of AMK (268.86°C). The onset and peak temperatures and enthalpy of fusion (ΔH) were similar for each sample. The SD and FD samples shown negligible differences in molar heat of fusion and peak temperature. The thermograms suggested that manufacturing process do not result into any interaction between components of ALLP and do not modify crystallinity of the drug.

SEM photographs of SD and FD-ALLP of TB and AMK revealed that SD-ALLP were dimpled spherical shape with roundness value of 1.048± 0.032, 1.066 ± 0.028 and elongation ratio of 1.206 ± 0.164, 1.263 ± 0.178 for TB and AMK respectively. However, FD- ALLP were irregular morphologies with large roundness value of 1.812± 0.338, 1.707 ± 0.454 and elongation ratio of 2.422± 0.984, 2.633 ± 1.084 for

TB and AMK respectively. Increased elongation ratio of FD -ALLP may be due to the growth of the longest axis of the crystals accelerated by process of sublimation of ice crystals from the frozen sample lattice. Topographical features of ALLP were derived from image analysis of SEM photographs suggests, varying degrees of surface roughness at different locations on surface of SD and FD-ALLP. The surface fractal dimension, which represents degree of particle surface corrugation, was determined from the texture of the images of SD and FD-ALLP surfaces. The results indicated that a significant ($p < 0.05$) reduction of roughness with SD-ALLP when compared to FD-ALLP. Higher value of heterogeneity and clumpiness for FD-ALLP further suggested high degree of roughness in contrast to SD-ALLP. These variations were probably related to differing in the processes and kinetics of particle formation. Controlled spray-drying process parameters, such as drying temperature, feed rate, aspiration volume etc., may have contributed to the formation of spherical and smooth particles.

The *in- vitro* aerosol performance of SD and FD-ALLP was determined using an eight stage, nonviable Andersen cascade impactor with a preseparator operating at an airflow of 28.3, L/min for 10 s. The percent mass distribution of SD and FD-ALLP on cascade impactor revealed that capsule and device retention of the FD-ALLP was significantly higher ($p < 0.05$) than those of SD-ALLP. The mass of powder delivered from the mouthpiece of the Rotahaler (emitted dose; ED) varied from 86-88% with SD-ALLP to 69-74% for FD-ALLP. The results indicated that 14-17% of recoverable dose of FD-ALLP was retained in the capsules and/or in the inhaler device. The reason for lower emission may be poor flow properties of FD-ALLP contributed by their traits such as large elongation ratio and irregular morphologies. The mass distribution data further suggested 10-12 % higher impaction loss for FD-ALLP than SD-ALLP. The lower impaction loss for the SD-ALLP when dispersed by the Rotahaler indicated that these particles were easier to disperse and the agglomerates formed during dispersion were much smaller than those of the FD-ALLP. It may be partly due to influence of traits such as large particle size, wide particle size distribution (high span value), and irregular morphologies with high values of elongation ratio and roundness. FD-ALLP were larger in size (as measured by MMADt) and possessed larger span: this means that there larger particles compared to SD-ALLP. This may be reason for high impaction loss due to deposition of FD-ALLP in upper stages of cascade impactor. The fine particle fraction (FPF) of SD-ALLP was

significantly higher ($p < 0.05$) than FD-ALLP, a better dispersibility of particles of < 5μm (stage 2 and the filter) was observed for SD-ALLP, 56-60% vs. 31-35%. Once a powder is dispersed, the FPF is determined by the aerodynamic diameter of the particles in the aerosol. Despite having significant difference in geometric size, both SD and FD-ALLP displayed marginal difference in aerodynamic diameters. The smaller aerodynamic diameter of FD-ALLP was because of higher drag force on the rough surface. The improvement of FPF with SD-ALLP was due to i) reduction in the capsule and device retention of the SD-ALLP and/or ii) increase in the powder dispersibility, that is, the amount of fine particles in the aerosol cloud per emitted dose. High FPF of SD-ALLP may also have been contributed by the spherical shape of SD-ALLP and lesser degree of particle surface corrugation. A little surface corrugation is reported to cause considerable improvement in the aerosol performance of the powder. Surface corrugation is known to decrease particle-particle interactions in two ways; the first, the asperities prevent close contact between particles and effectively increase the inter-particle distance and the second, the asperities reduce the total area accessible for interaction. Another possible reason for improved aerosol performance may be lower electrostatic charges on ALLP produced by spray drying. The reason for higher electrostatic charges on FD- ALLP may be due to size reduction of lyophilized cake.

ALLP produced by spray-drying technique was found to have better aerosol performance than FD-ALLP. Traits and topographical features of ALLP, such as particle size, polydispersity, elongation ratio, roundness, shape, and degree of surface roughness (surface corrugation) were found to be significantly influenced by the technique of production. SD-ALLP was shown to possess higher FPF, lower impaction loss, and less capsule and device retention. Hence, the carefully selection and optimization of method of preparation will significantly improve the deposition of drug to the lower airways needed in CF and non- CF bronchiectasis diseases. SD-ALLP had been selected to further investigate the quantitative aspects of the effects and relationships among various formulation components of high therapeutics payload ALLP, which are profoundly influenced by several formulation components.

For the optimization process sixteen batches of ALLP were prepared varying three independent variables (percent weight by weight of L-leucine (X_1), TB/AMK(X_2) and poloxamer-188 (X_3) of the formulation. The influence of these variables on observed response (Y, fine particle fraction) was recorded. The maximum responses were

62.47% & 66.24% and minimum responses were 40.57% & 37.44% for TB and AMK-ALLP respectively. The mathematical relationship for TB and AMK in terms of a polynomial equation relating the response Y and independent variables was: $Y= 56.2068 + 5.7481 X_1 - 3.0531 X_2 + 0.8468 X_3 + 1.1737 X_1 X_2 - 0.5012 X_1 X_3 - 0.7412 X_2 X_3 - 0.7149 X_1^2 - 1.9212 X_2^2 - 1.6187X_3^2$ and $Y= 53.464 + 7.137 X_1 - 2.112 X_2 + 2.013 X_3 + 0.707 X_1 X_2 - 2.149 X_1 X_3 - 2.265 X_2 X_3 + 0.278 X_1^2 - 2.061 X_2^2 - 0.998X_3^2$ respectively. Equations express the quantitative effect of the individual formulation components (X_1, X_2, and X_3) and combination thereof on the response (Y) in terms of interaction coefficients. The values of the coefficients X_1 to X_3 are related to the effect of these variables on the response (Y). Coefficients with more than one factor term and those with higher order terms represent interaction terms and quadratic relationships respectively. A positive and negative signs suggest a positive and negative effect on response respectively. The theoretical (predicted) values and the observed values were in reasonably good agreement.

The significance of the ratio of mean square variation due to regression and residual error was tested using analysis of variance (ANOVA). The ANOVA indicated a significant ($P < 0.05$) effect of factors on response. Lack of fit was not significant ($p = 0.08$, $p = 0.13$) and regression was strongly significant ($p = 0.01$, $R^2 = 0.92$; $p = 0.001$, $R^2 = 0.96$), so it was concluded that the second-order model adequately approximated the true surface. The estimated second-order RSM indicated that TB/AMK proportion affected negatively, while the L-leucine and poloxamer -188 proportion was affected positively. Removal of L-leucine from the formulation greatly affect aerosol performance, poloxamer -188 at higher levels did not have significant effect on response Y and proportion of TB/AMK and poloxamer -188 at lower levels did not affect response Y. L-leucine was found to be most influencing component and increase in TB/AMK proportion above 50% w/w produced dense particles leading to poor aerosol performance. While, poloxamer-188 found to influence aerosol performance at low concentration (1-2 %w/w). After achieving a polynomial equation and independent variables (X_1 to X_3) were optimized for the maximum response (Y). ALLP containing a high drug (TB/AMK) load of 44.17 and 47.85 % w/w were prepared by spray drying technique using L-leucine/poloxamer- 188/HSPC. The maximum fine particle fraction of 62.8 ±2.6% and 64.17±2.6% was obtained with L-leucine/TB/poloxamer-188/HSPC at 20/44.17/1.19/34.64 w/w/w/w and L-leucine/AMK/poloxamer-188/HSPC at 20/47.85/1.0/31.15 w/w/w/w of ALLP

respectively. Particles were dimpled spherical shape with roundness value close to 1 and were having smooth surface texture with good powder dispersibility suitable for use in carrier-free DPI. At predicted levels of X_1, X_2, and X_3, the obtained response (Y) was found to none significantly ($P< 0.05$) different from the predicted value. This demonstrated the reliability of the optimization procedure in predicting the aerosol performance of ALLP containing high therapeutic payload.

Influence of various important factors like humidity and airflow rate on aerosolization of ALLP was investigated. Flow and dispersion characteristics of ALLP were also studied. In order to understand the effect of humidity on the ALLP, particles were characterized in terms of particle size and dispersibility (span value). VMD of SD TB/AMK ALLP subjected to varied humidity conditions revealed non significant ($P>0.05$) particle growth at 15-45% RH. However at 60 and 75% RH, marginal increase of VMD and 'span' was observed, but was well with in the respirable range.

Analysis of the Karl Fischer water contents suggested a significant (ANOVA $p<0.05$), positive increase in water content, for both ALLP, at storage conditions of 60%RH and above. The high moisture sorption above 60%RH partly may be because of large proportion of hydrogenated soyaphospatidyl choline (>30%w/w) in the formulation. In addition to this, the crystalline nature of both TB and AMK would have further contributed to the high moisture sorption. As discussed earlier (DSC thermograms), the crystallinity of both spray dried TB and AMK-ALLP do not altered by manufacturing process. Crystalline drug molecules staked in rods, held together by water molecules, on high humid conditions considerable moisture sorption into the crystal lattice can occur.

The *in vitro* characterization of the aerosolization of TB and AMK-ALLP was conducted at 15, 30, 45, 60, and 75% RH by using cascade impactor. The capsule and device retention was significantly higher ($p < 0.05$) above 60% RH, with respect to humidity conditions of 15-45% RH. The mean percentage delivered dose (ED) falling from 89% at 15% RH to 74% at 75% RH with TB-ALLP and 91% at 15% RH to 73% at 75% RH with AMK-ALLP. The results indicated that 15 and 18% of recoverable dose of TB and AMK-ALLP was retained in the capsules and/or in the inhaler device at high humidity conditions. The reason for lower emission may be due to formation of agglomerates at high humid conditions. A plot of FPF against humidity, suggested a decrease in mean FPF of both ALLP, at 60% and 75% RH, which may be attributed to the large increase (about 3-4% w/w) in moisture uptake above 60% RH. The

relationship between increased humidity and decreased aerosolization performance of ALLP most likely is attributed to the hygroscopic nature of the powder, because water is rapidly absorbed into the crystal lattice at a specific humidity until it forms equilibrium with the surrounding environment. The presence of such a dynamic equilibrium can only promote the condensation of water between the capillaries of the powder particulates, thus increasing the inter-particulate forces while decreasing the aerosolization efficiency.

Influence of airflow on the dispersion behavior of ALLP was investigated in cascade impactor, operated at airflow of 28.3, 60 and 90 L/min for 10 s, 5 s and 3 s respectively. ALLP were almost completely dispersed (mean value of 94.26±1.81 and 92.45±2.78 for TB and AMK ALLP respectively) from the Rotahaler at all above air flow rates. The amount of ALLP emptied was similar at all air flow rates. The MMADe of the ALLP were 4-5 μm at air flow rate of 28.3 L/min to 90 L/min, and were larger than theoretical estimates of primary aerodynamic diameters, indicating that the powder aerosols exited the inhaler device as particle aggregates and that increasing the airflow rate created insufficient differences in shearing to profoundly impact on the degree of dispersion of these aggregates. This is advantageous for pulmonary drug delivery because it alleviates the dependence of delivered doses with the breathing pattern of the patient.

Evaluation and control of flow and dispersion (deaggregation) characteristics of the formulation are of critical importance in the development of DPI products. The flowability and floodability expressed by angle of repose (28.2 ± 0.4 and 30.2 ± 0.3), Hausner ratio (1.38±0.01 and 1.26±0.03), dispersibility index (20.7 ± 0.2 and 20.2 ± 0.4), and compressibility index (24.6 ± 2.8 and 23.3 ± 1.8) for TB and AMK-ALLP respectively were under the category of good and floodable. Packing properties of the ALLP were determined using the tapping method using Kawakita's equation. There was a linear relationship between '**n**' and **1/(εn-εf)**, and the constant ***K*** obtained from the slope of this line was comparable with respect to the reference Inhalac 230.

The stability studies were carried out as per ICH guidelines for countries falling under zone III (hot, dry) and zone IV (very hot, humid). The product in its final packing (gelatin capsule in HDPE bottles with PVC coated aluminum foil cap) was stored separately for each sampling point and evaluated for particle size, assay, degradation, water content, ED and FPF. The results of stability data revealed formation of small agglomerates in the both TB and AMK-ALLP after storage period of 3 months under

accelerated conditions. There was no *"significant change"* observed in the assay content of both formulations. No potential degradants also observed during their entire storage period at all conditions. Analysis of the Karl Fischer water contents suggested a significant (ANOVA $p<0.05$), positive increase in water content at 3M/40±2°C/75±5%RH. The moisture content in ALLP increased from 3.06 ± 0.12 to 5.16 ± 0.41 and 3.46 ± 0.19 to 5.27 ± 0.34 for TB and AMK- ALLP respectively. The increased moisture level of ALLP may responsible for formation of small agglomerates in the formulation at 3M/40±2°C/75±5%RH. The high moisture sorption of ALLP at accelerated condition (40±2°C/75±5%RH) partly may be because of large proportion of HSPC (>30%w/w) in the formulation. In addition to this, the crystalline nature of both TB and AMK would have further contributed to the high moisture sorption.

There was positive increase in particle size growth of ALLP at storage period of 3 months under accelerated conditions. The VMD of ALLP increased from initial value of 6.75 ± 0.23 to 9.19 ± 0.12 for TB-ALLP and 6.82 ± 0.33 to 10.13 ± 0.11 for AMK-ALLP at 3M/40±2°C/75±5%RH, which are about 36% and 48% increase in particle size growth for TB and AMK ALLP respectively. However the altered particle size is still fits in the respirable size window of 5-30μm for ALLP.

"Significant change" in the functionality tests such as ED and FPF of ALLP were observed at 3M/40±2°C/75±5%RH. The capsule and device retention was significantly higher ($p < 0.05$) on 3rd month onwards under accelerated condition. The mean percentage delivered dose (ED) decreased from 86.72 ± 1.18 to 78.57 ± 2.07 and 91.47 ± 2.26 to 76.54 ± 1.28 for TB and AMK-ALLP respectively. The results indicated that about 8 and 15% of recoverable dose of TB and AMK-ALLP was retained in the capsules and/or in the inhaler device at 3M/40±2°C/75±5%RH. The reason for lower emission was due to formation of agglomerates at high humid conditions. A plot of FPF at different time intervals suggests a decrease in mean FPF of both ALLP from 3rd month onwards at 40±2°C/75±5%RH was due to the increase (about 2% w/w) in moisture uptake. The relationship between increased humidity and decreased aerosolization performance of ALLP most likely is attributed to the hygroscopic nature of the ALLP.

The ED and FPF of both TB and AMK-ALLP at 40±2°C/75±5%RH were below the acceptable level as per the ICH guidelines. Hence, shelf life can not be assigned, until the formulation meets the criteria at intermediate conditions. Hence, formulations

were tested on intermediate storage condition in order to assign a shelf-life at CRT. The product stability in terms description, chemical stability, particle size growth, moisture content and in-vitro deposition characteristics were conducted for one year at intermediate storage condition. No significant change was observed in the powder properties at all sampling points. Based on the results of intermediate condition and real time study data both TB and AMK-ALLP can ascertain shelf life of 12 months as per the ICH and USFDA at CRT. The shelf life can be further re-ascertained base on the real time study data at 25±2°C/60±5%RH.

In vivo pulmonary deposition, pulmonary clearance, alveolar macrophage uptake and pulmonary toxicity of TB/AMK-ALLP were evaluated in rat and compared with their respective lactose base formulations. Deposition is an important parameter for any inhaled drug, and is of critical importance for drugs such as antibiotics and anti-infectives meant for local action. Estimation of regional distribution of inhaled antibiotics would give the true picture of deposition pattern at target site. To understand the site of deposition of ALLP, regional distribution was assessed and compared with conventional lactose base formulations (LBFs) with in the lungs. For assessment of regional lung deposition, the lung was divided into trachea (with main bronchi) and pulmonary lobes. Each lobe was cut in two equal parts by mass: one central part cut round in shape around the bronchus hilum, and one peripheral part; and the different tissue samples were finely minced and drug content was estimated. To compare the efficiency of deposition of the different formulations in the lung, several parameters were measured. (1) The deposition in the trachea (T), central (CS), and peripheral (PS) lobe sections was expressed as percentages of total deposition within the lungs. Because the proportion of lung parenchyma (the preferential site of absorption to the systemic circulation) to conducting airways is larger in the peripheral lobe section compared to the central lobe region, the ratio of deposition in the peripheral to central lung (which included the central lobe section and the trachea; P/C ratio) was used as an index of deep lung deposition and was calculated as [PS/ (CS+T)]. A P/C ratio close to 1.0 indicates a homogeneous deposition within the lung lobes and limited deposition in the trachea, whereas a ratio close to 0.0 indicates a preferential deposition in the trachea and the central lobe section.

Significant difference ($P<0.05$) was observed in the percentage deposition of pattern of ALLP and LBFs. For both TB and AMK-ALLP, almost 53-58% was found in the right and left lungs against 22-26% with LBFs. The percentage of central deposition

relative to total deposition reached 31.70±1.26%, 31.75±2.27%, 16.31±1.18 % and 14.05 ±1.58 % for TB-ALLP, AMK-ALLP, TB-LBF and AMK- LBF respectively. The corresponding P/C ratios were 0.43±0.06, 0.44±0.05, 0.17±0.05 and 0.15±0.04 respectively. TB and AMK-ALLP shown superior aerosol performance and more homogenous deposition with in the lung with respect to their LBFs. Central lobe region had high deposition fraction as compared to peripheral region. High deposition fraction in the central lobe region may reduce the therapeutic dose of antibiotics and anti-infectives intended for local action. Because, central lobe region is rich in conducting airways and is the target site for treatment of diseases involving infection of the airway (CF, non CF-bronchiectasis).

High FPF of the ALLP measured *in vitro* translated into significant respirable fractions *in vivo* in the rat lung. The fraction of particles with an aerodynamic size <5 μm was 62–64% in the eight-stage cascade impactor using a Rotahaler device at an airflow rate of 28.3 L/min. The total fraction of the delivered ALLP mass that was recovered from the lung lobes reached a relatively close value of 53-58%. It is noteworthy that dry powder dispersion and penetration in the lungs were not dramatically impeded by the high relative humidity of the respiratory tract animals.

The lung concentration of TB/AMK remained high for long period of time with ALLP formulations. Over the 4 h following administrations of LBFs the drug load in the lung decreased to about 23% of the initial drug load. After 4 h, the levels of TB/AMK in the lung tissue were below the limit of quantification. In contrast when TB/AMK was administered as ALLP, the level of TB/AMK remained well above quantifiable level for at least 12 h of post administration, indicated reduced pulmonary clearance of drug from ALLP. It is possible that the maintaining of high level of antibiotic concentration over sustained period may be partly due to the more slowly clearance of ALLP, because of the role of large particles in escaping phagocytosis. This indicated that the delivery approach may require infrequent dosing to achieve the desired clinical effect. In addition, this retained pulmonary antibiotic concentration may also reduce the potential buildup of resistance due to the reduced need for patient compliance in maintaining high levels of drug over time. This profile of increased lung antibiotic concentration over a sustained period of time could be advantageous in reduction or prevention of selective bacterial resistance when using aminoglycosides, as this type of antibiotic exhibits concentration-dependent killing

with time. In fact, dosing regimens of targeting high peak concentration relative to MIC appear to yield the best clinical outcome.

Alveolar macrophage uptake study revealed that, after 1h of administration 17.82 ±4.3% of phagocytic cells contained TB-LBF and 21.6 ±3.2% contained AMK-LBF. By contrast, only 11.5 ±3.4% of phagocytic cells contained TB-ALLP and 9.63 ±3.5% contained AMK-ALLP. For LBF, 19.77±2.5 % (TB-LBF) and 24.8±3.7% (AMK-LBF) of the phagocytic cell population contained numerous particles 1h after inhalation, compared with 5.86± 1.8% (TB-ALLP) and 4.62±1.3% (AMK-ALLP) for ALLP. These results were consistent with earlier findings that phagocytosis of particles diminishes precipitously as particle diameter increases beyond 3 μm. LBFs shown evidence of a numerous particles within the cell cytoplasm, whereas reduced phagocytic uptake (0-2 particles per cell) was observed with ALLP. Maintaining of high level of antibiotic concentration over sustained period is desired for the reduction or prevention of selective bacterial resistance, it must be retained in the lung for a prolonged period .When foreign particulate material reaches the alveoli, where no ciliated epithelium is present, deposited particles may stay for longer times. However, in the alveolar region of the lung, microparticles come into contact with another lung defense mechanism, the alveolar macrophages. Therefore, to enable such prolonged lung antibiotic concentration requires the ability of particles to escape detection and up-take by alveolar macrophages.

Pulmonary toxicity was assessed by analysis of cellular and fluid components of bronchoalveolar lavage fluid (BALF). The parameters examined were total proteins, indicative of transudation of serum proteins across the capillary barrier, lactose dehydrogenase (LDH) indicative of general cell injury and acid phosphatase (AP), a lysosomal enzyme which is released during the phagocytosis and/or macrophage and neutrophil damages. If increased level of these enzymes detected, indicates cell injury. There was no significant difference ($P>0.05$) in the cell recovery, whether macrophages, neutrophils or eosinophils. This was similar with sham operated animals, and those who inhaled TB/AMK LBF, TB/AMK-ALLP. Similarly, there was no significant difference in total protein (about 23 mg for sham operated, 21-26 mg for treated animals), AP (about 16 units for sham operated, 14-16 units for treated animals) and LDH (about 102 units for sham operated, 97-100 units for treated animals) levels. These suggested that inhalation of either LBF or ALLP do not

produce either pulmonary inflammation or lung cellular injury. The effects of ALLP and LBF on morphological integrity of respiratory tract were assessed. Tracheal and lung sections from animals dosed intra-tracheally shown normal morphology indicating that the intra-tracheal dosing procedure did not cause any physical disruption. Moreover, no pathological changes were observed in the trachea and in the lung over the time course of experiments.

Findings of these studies conclusively demonstrated superiority of ALLP formulations over conventional LBFs by exhibiting high FPF, homogenous pulmonary deposition, drug concentrations in the lung tissues for prolonged time, slow clearance ALLP from the lung and reduced toxicities.