DEVELOPMENT OF A TECHNOLOGY PLATFORM FOR TRANSIENT TRANSFECTION OF LUNG AND AIRWAY EPITHELIUM WITH CYTOKINE GENES AS AN ALTERNATIVE TO ADMINISTERING RECOMBINANT PROTEINS FOR TREATMENT OF LUNG CANCER AND OTHER INDICATIONS

Therapeutic and prophylactic interventions based on immune modulation use proteins and genetically engineered cells to modulate immune mechanisms that can prevent and combat disease (1). Vaccination, passive antibody therapy, CAR T-cells, stem cells and cytokine therapy have demonstrated that immune modulation can be effective and safe(2). Gene therapy, however, is currently not in extensive clinical use. This is because the dominant objective of gene therapy is to introduce desired genes into autologous cells that will last a lifetime. It is not feasible to undertake such a clinical procedure repeatedly. Yet, in contrast to stable transfection, transient transfection of mammalian cells with plasmid DNA is facile, in vitro and in vivo. Transient transfection, by definition results in expression of the gene of interest for a limited period of time, and has not received serious attention.

We have earlier demonstrated the preclinical proof-of concept of the efficacy and therapeutic benefit of transient transfection with gamma interferon in a mouse model of tuberculosis (3,4). This proposal describes the developmental pathway and refers to Drug Regulatory challenges in respect of a non-invasive, self-administered, non-sterile, room temperature stable, formulation of dry powder inhalations (DPI) of plasmid DNA. As an example, it describes a potential alternative to Actimmune®, which is in clinical use for several decades. This proposal is submitted for the award of a short-term fellowship, but the intent of the applicant is to seek a career with Sun Pharma to develop the proposed technology platform—a process that will take 5-7 years. Progress made in the period of the project will enable evaluation of the feasibility and pharmacoeconomics of developing a pharmaceutical product for marketing globally.

The immune system is complex—optimally, it must identify microbes and the body's own rapidly dividing cells and respond to those that can cause harm; that too in a manner that ensures the elimination of the threat while minimizing damage to the body. Timing is a key feature to ensure this. Thus, pharmacokinetics and disposition of the expressed human gene need to be carefully engineered. Optimally, immune response to infection and neoplasm is rapid, efficient, and self-limiting. Successfully stimulating the correct immune pathways is vital for resolving infections, but it is equally important to downregulate the immune response once the infection is cleared, as

overstimulation can lead to harmful, uncontrolled inflammation. The transient nature of gene expression is the key feature that differentiates the proposed DNA therapeutic, both from gene therapy and cytokine (protein) therapy. The proposal has scope to create intellectual property (IP) in the field of immunotherapy: a fast-expanding discipline with the potential to develop innovative medicines for cancer, autoimmune disorders, and infectious diseases(5).

Actimmune® (interferon-gamma-1b) is a biological product used primarily to treat chronic granulomatous disease (CGD) and severe malignant osteopetrosis (SMO). CGD is a genetic disorder, usually diagnosed in childhood, that affects some cells of the immune system and the body's ability to fight infections effectively. CGD is often treated (though not cured) with antibiotics, antifungals, and Actimmune® (6). SMO is a genetic disorder that affects normal bone formation and is usually diagnosed in the first few months after birth. While effective, Actimmune® has several patient access issues (6). The treatment is expensive, with annual costs reaching hundreds of thousands of dollars and requires specific administration conditions, including regular injections and monitoring, which can be burdensome for patients and healthcare providers (7).

Direct delivery of host-directed therapy (HDT) to the respiratory tract and lungs is, from first principles, more effective than systemic administration in reaching the airway and lung mucosa (8). Interferons (IFNs) are host proteins that act in an autocrine and/or paracrine manner to influence defence responses. For clinical use, IFNs are prepared as recombinant, injectable, sterile products; under Good Manufacturing Practices (GMP). Being extremely unstable, they require a cold chain up to the point of care. Inhaled IFN-β protein underwent clinical trials for management of COVID-19(9)at the asthma-recommended dose level of 6 mIU(10)for 14 days, by nebulization. Nebulized IFN-γ has demonstrated benefit in patients of multi-drug resistant pulmonary tuberculosis, caused by an intracellular bacterial pathogen (11). Nebulization is not preferred for delivery of medicaments to the lower respiratory tract and lungs. Scaling-up, storage, transport at 2-8°C; and deployment of sterile, parenteral, recombinant, glycosylated protein products is cost-intensive.

In limited-resource settings, deploying recombinant proteins for management of pandemics appears infeasible. However, the use of 'transient' gene therapy may be scaled to hundreds of thousands of patients with reasonable cost. This project will test the hypothesis that *transient*

transfection of the lung and airway epithelium to produce the protein of interest for an appropriate duration, will activate host responses that are favourable to the host to fight against infectious and neoplastic diseases of the lungs and airways, without inducing immunopathology in the host tissue.

The DNA construct to be delivered requires careful thought. It would be naïve to deliver an IFN gene under a strong promoter for long-term expression, as would establishment of long-lasting concentrations of IFNs by protein administration. Because cytokine secretion responses are self-amplifying in nature, care is required to ensure that IFN responses generated by exogenous means are not amplified to "cytokine storm" levels. Immune homeostasis must only be "tweaked" and not "driven" if the safety of the interventionis to be maintained. Exposure to IFNs, therefore must be **transient** and guided by **dose-limiting immunotoxicity**.

DPI bearing plasmid DNA as a differentiated formulation from Actimmune®

The active pharmaceutical ingredient (API) in the proposed formulation is a nucleic acid, whereas Actimmune® employs a protein. This level of differentiation also creates scope for generating IP. Other competitive advantages of the DPI include the low cost-to manufacture compared to Actimmune® and the following considerations:

1. *Non-Sterile Product*: Unlike Actimmune®, which requires a sterile liquid formulation for injection, DPI formulations are not required to be sterile (the air we breathe is non-sterile). This significantly reduces manufacturing costs, as the stringent and expensive asceptic processing or terminal sterilization processes required for injectable formulations are unnecessary for DPI.
2. *No Need for Cold-Chain Logistics*: The DPI formulation is stable at room temperature, eliminating the need for cold-chain logistics. Actimmune, on the other hand, requires refrigeration throughout its storage and transport, adding complexity and cost to its distribution. The room-temperature stability of the DPI formulation makes it not only more affordable but also more accessible, especially in regions with limited infrastructure.
3. *Potential for Self-Administration*: The DPI formulation can be self-administered by patients using a simple inhaler device, reducing the need for frequent healthcare visits. This contrasts with Actimmune®, which requires subcutaneous injections that need to be administered by healthcare professionals. The ability to self-administer the DPI reduces

both direct healthcare costs and the indirect costs associated with time off work or travel for medical appointments.

These factors combined make the DPI formulation a more economical option for delivering genes of interest for transiently transfecting the lung and airway epithelium, potentially offering substantial savings for patients, and healthcare providers as well as returns on investment for the innovator. The following Sections describe the steps already standardized and how they will be impemented for the present proposal.

GMP-compliant production of API at Pilot Scale

The plasmid pDsRedExpress-N1 was procured from Clontech (USA), while pCMV6-Entry Vector (MC 208752) and pCMV6-AC-GFP vector (MG227155) with the mouse IFN-g gene in tandem with the reporter gene (green fluorescent protein, GFP) were obtained from Origene Technologies, Inc., USA. The plasmid was transformed into competent *E. coli* DH5-α cells using 2ng of each expression plasmid. Heat shock was given by incubating the DNA with cells at 42^{o}C for exactly 30 seconds before immediately putting the cells on ice. Luria-Bertani (LB) broth (250 µL) was added to the cells and kept at room temperature (RT) for 5 minutes, after which the tubes were incubated at 37^{o}C for 1 hour with agitation.

In-process Quality Control (IPQC)

Dilutions of the transformation mixture (1 percent, 10%, and 50% in LB medium, each diluted to 100 uL) were plated onto different agar plates (LB-amp, 100 ug/mL ampicillin in LB medium) and incubated overnight at 37 ° C. The next day, 2-3 isolated bacterial colonies that had grown on the plates were inoculated into separate sterile culture tubes containing 5 mL of LB-amp broth from each transformation. These were incubated at 37°C with agitation (200–250 rpm) overnight. The optical density (OD) of the cells at 600 nm was tested frequently for isolating amplified plasmids until it reached between 0.6 and 2.

Downstream Processing to Recover Plasmid DNA

To pellet down the bacteria, centrifugation at 5000*g for 15 minutes at 4°C was done. The pellet was resuspended in buffer (50 mM Tris-HCl, 10 mM EDTA, 100 g/mL RNase A, pH 8.0) after the supernatant was removed. A suitable amount of lysis buffer was added to the bacterial suspension: 200 mM NaOH with 1% SDS. After 2 minutes, renaturing buffer (2.8 M KAc, pH 5.1) was added to the denatured bacterial culture. To isolate the genomic DNA, the denatured bacterial

culture was centrifuged at 12000*g for 25 min. The bulk of cellular proteins were removed with the supernatant. Isopropanol was added to precipitate the plasmid DNA. The precipitated DNA in isopropanol suspension was added to a column (NucleoBond® AX 100 Columns, silica-based anion-exchange resin developed by Macherey Nagel). The column was washed with 70% ethanol and the DNA eluted from column by elution buffer (5Mm Tris/HCl, pH 8.5).

Tests for Identity, Assay, Purity Stability and Related Substances

The yield of plasmid preparations (*Assay*) was calculated from the ratio of absorbance at 260 and 280 nm (A260/A280) using a Nanodrop spectrophotometer (Thermo Scientific), which also indicated the presence/absence of *related substances*. The *identity*, *purity* and *related substances* in the DNA were established by running the samples on a 1 % agarose gel for the conformation and structural integrity (*stability*) of isolated plasmid DNA. Restriction digestion with the enzymes XhoI and HindIII was done to confirm the gamma interferon gene insert in the plasmid (*identity*). Insert and plasmid bands were separated and visible under gel electrophoresis (Figure 1).

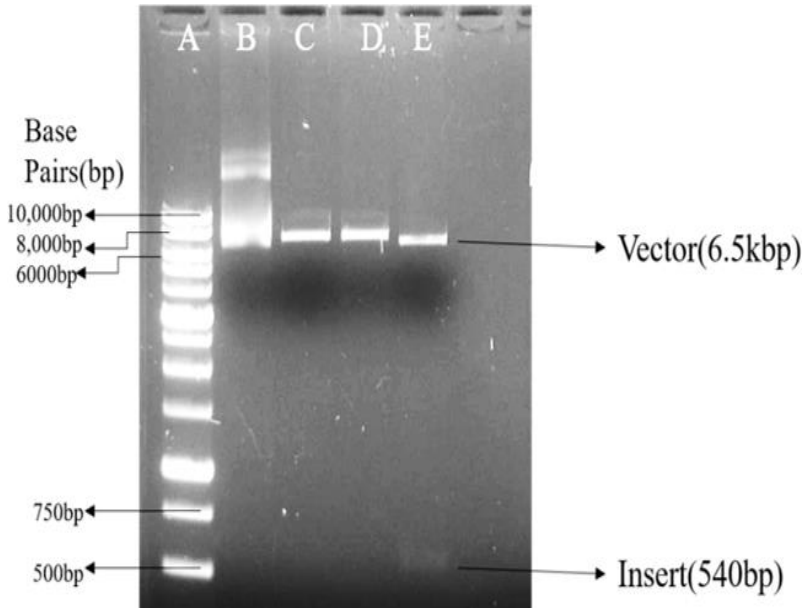

Figure 1: Restriction digestion of pDNA followed by analysis on 1% agarose gel. *Lane A*: 1kb DNA Marker; *Lane B*: Native Pcmv6-AC-GFP (6.6 kbp) pDNA; *Lane C*: single digestion with XhoI; *Lane D*: Single digestion with EcoRI; *Lane E*: Double digestion with EcoRI and XhoI.

Formulation Step 1: Polyplex formation

We used *poly(*ethylenamine) or PEI to make a stable polyplex with DNA. The details of the excipient are not disclosed here, and are crucial for preparing an optimal formulation. We determined the affinity of PEI for DNA for preparation of a stable polyplex. The least amount of PEI required to make a stable polyplex with DNA was investigated. Plasmid DNA incubated with PEI in nucleotide: polymer (N/P) ratios ranging from 1:2 to 1:20 and then agarose gel electrophoresis was performed and observed that even the smallest proportion of PEI to DNA of 2:1 was suitable to make a stable polyplex. We observed that the supercoiled plasmid with a molecular weight of 6.6kbp was not released from the polyplex. indicating that N/P at a ratio of 2 is sufficient to form a polyplex (Figure 2, Left Panel). The DNA from the polyplex was displaced by incubation with heparin. Heparin binds DNA with greater avidity than PEI (Figure 2, Right Panel).

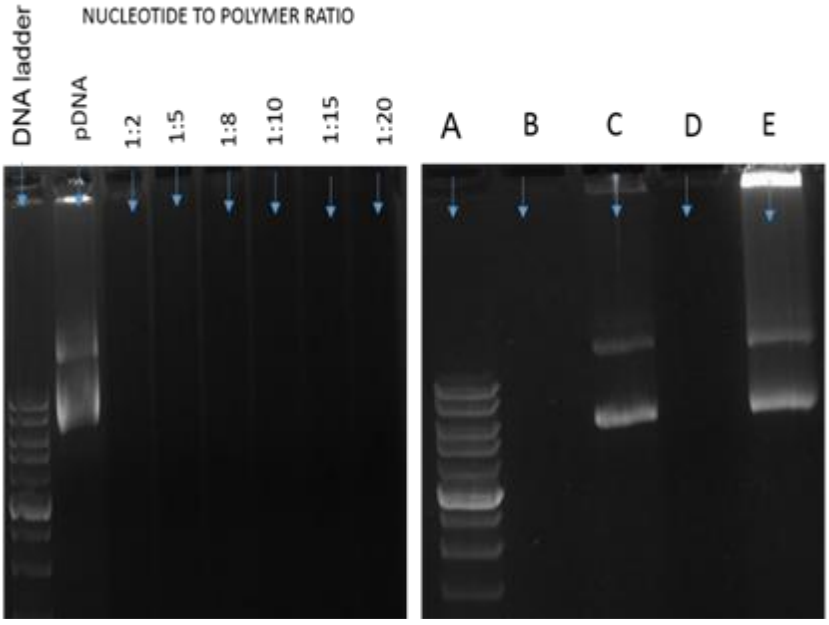

Figure 2. Gel electrophoresis. (*Left*): N/P of 1:2 is sufficient to ensure that DNA in polyplexes does not migrate electrophoretically under conditions that induce migration of free plasmid DNA. (*Right*): DNA retains structure after spray-drying. (*Lane A*): 1 Kb DNA ladder, (*B*): DNA-polymer polyplex, (*C*): plasmid DNA, (*D*) DNA in spray dried powder, (*E*): DNA released from spray-dried particles by heparin.

Formulation Step 2: Spray-Drying

We used spray drying to prepare particle of prepared polyplex. Excipients were screened and spray-drying parameters were optimized to prepare the formulation suitable for deep lung deposition. Dry powder formulations suitable for pulmonary delivery were prepared employing optimized conditions to obtain formulations with good flow characteristics, proper particle size, and minimal hygroscopicity. The optimized formulation comprised standardized weight % of β-cyclodextrin, lactose and L-leucine. The excipient solution was prepared in 15% ethanol. The PEI solution of 1mg/ml was prepared and mixed with plasmid in optimized ratio of N/P(1:2). The solution of PEI and plasmid (PEI: DNA) was kept at rest for half an hour. In distilled water/ethanol (85:15 v/v), the PEI: DNA polyplex was combined with excipients, and the solution was spray dried (Buchi B-290 Mini-Spray-Dryer, Buchi Labortechnik AG, Switzerland). An inert loop was employed to spray the solution with two fluid nozzles. Using standardized parameters of inlet temperature 110°C, spray feed rate 5mL/min, aspirator setting 85 percent, and pump setting 16 % in a controlled environment (30% RH and 25°C), the output temperature observed during spray drying was 63ºC.The resulting products were collected and characterized. Figure 3 shows a diagrammatic representation of a spray dryer and its essential components (12). IPQC was carried out by recording inlet and outlet temperature readings at 10 min intervals. A Batch Production Record (BPR) and SOP was developed for the standardized process.

In the present proposal, a process of manual capsule-filling will be developed and optimized to fill DPI capsules compatible with an inhaler device and the performance of the powder in a selected inhalation device for human use will be optimized,

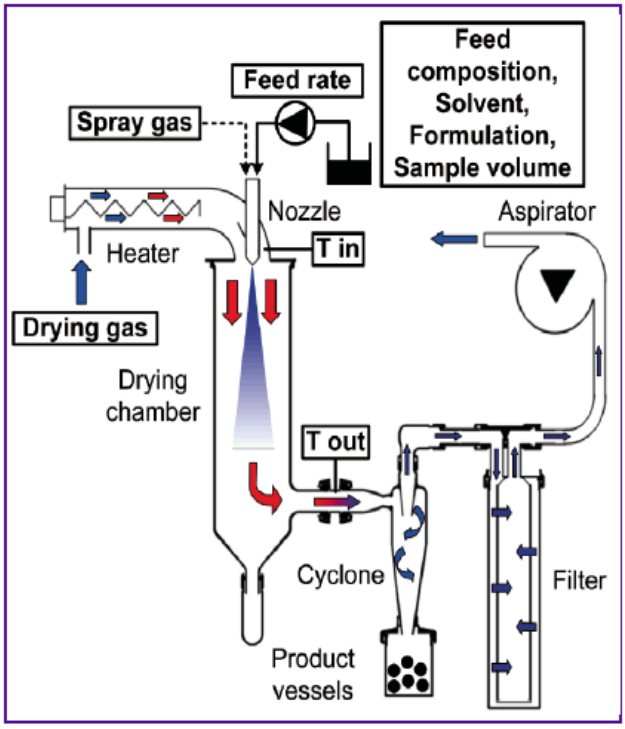

Figure 3. Diagrammatic representation of Spray Dryer and its essential components

Standard Testing Protocol (STP) and Batch Release Specifications (BRS)

The STP and BRS of the DPI (without capsule filling) was developed, and is ready for immediate implementation to the present project.

Assay, Uniformity of Unit Dose Content and Storage Stability

After extracting DNA from DPI particles using the conventional phenol-chloroform-isoamyl alcohol technique, agarose gel electrophoresis was done to check the stability of DNA in prepared formulation (Figure 2, Right Panel) and quantified using the Nanodrop (Thermo Scientific, Nanodrop 2000c). The ratio of absorbance at 260 and 280nm was 1.81± 0.01. The DNA content in the powder was estimated as 5.45 µg/10 mg, i.e., 0.0545% w/w.

Surface morphology

The surface characteristics, particle shape, and self- aggregation of particles was observed under a scanning electron microscope (Tecnai, Phillips). Scanning Electron Micrographs of the spray dried particles showed these to be spheroidal in shape, with several particles bearing large pits. Occasional fibers and dendritic projections from the particle surface were observed (Figure 4 A,

B). Pitting and cratering of the particle surface is likely due to sudden evaporation of solvent from "bubbles" on the drying surface, resulting in caving-in of the ruptured surface. Dendritic processes are likely to have arisen from bridges between drying droplets that break as the forming particles move apart(13,14). Particle size distribution was established by measuring the Martin's diameter of a total of 264 counts in the two fields of the SEM micrographs shown in Figure 5

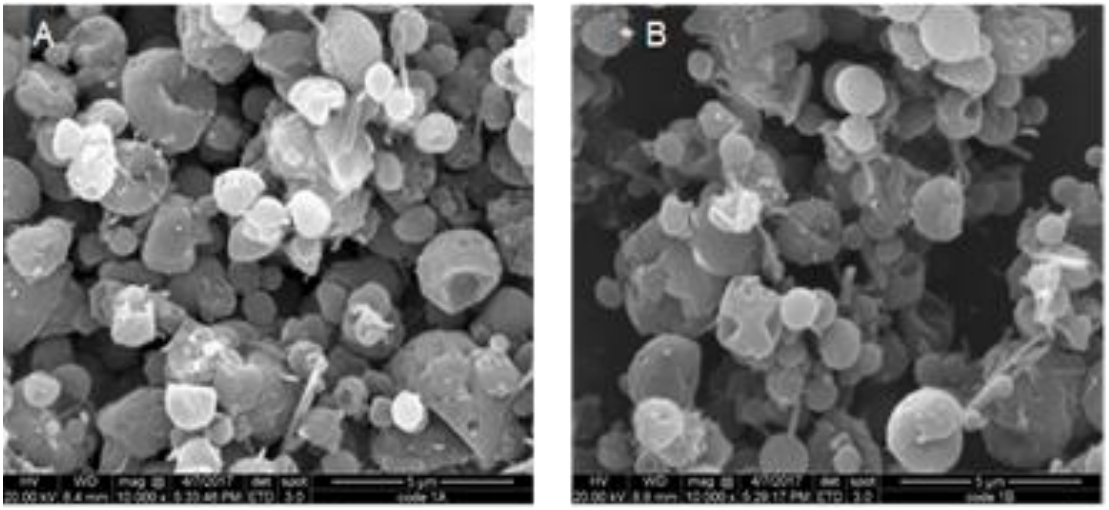

Figure 1 (A, B): Scanning electron image of the spray dried particles from two representative batches. The scale bar is 5µm.

Particle Size Distribution by Microscopy

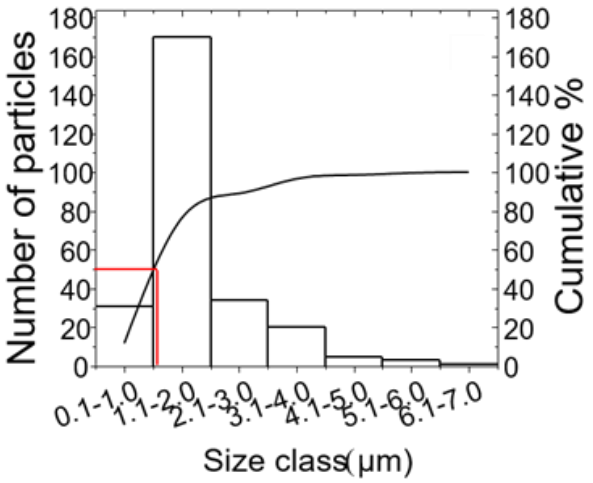

Figure 2 Particle size distribution of the Martin's diameter of particles visualized by SEM. The cumulative frequency curve was used to find the intercept of the size corresponding to 50% undersize as the median diameter.

Particle Size in Hexane Suspension by Dynamic Laser Scattering

The size of spray dried particles was determined by DLS (Mastersizer 2000; Malvern Instruments, Malvern, UK). About 50 mg spray-dried powder was dispersed in 20 mL hexane containing 0.5% w/v Span 80 and the required laser beam obscuration of >15% under sonication was achieved. The span volume-average diameter of particles was estimated relative to the median diameter (D[υ,50]) was derived from D[υ,90]-D[υ,10]/D[υ,50], where D_{50} is particle diameter at 50% cumulative size, $D_{90}$is particle diameter at 90% cumulative size; and $_{D10}$ is particle diameter at 10% cumulative size. The median size of dry particles was about 1.5 µm (Figure 6).

The difference between the diameter calculated by SEM and DLS is due to the hydration of the particles when suspended in hexane. However, the preparation was monodisperse, with >90% of particles covered in six size classes between 7 and 11 microns.

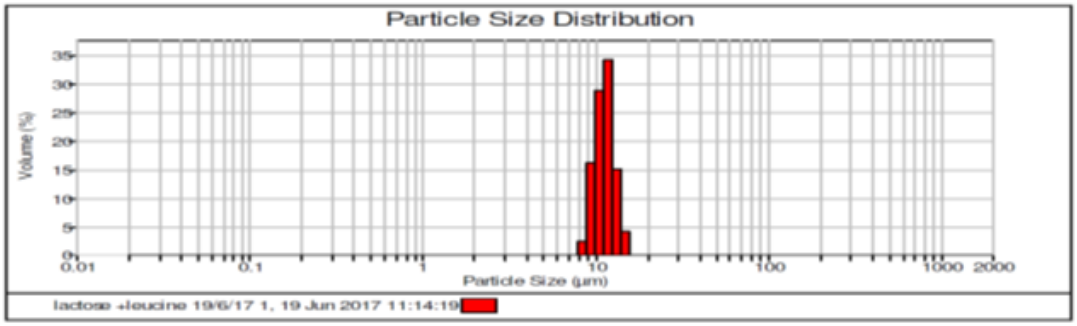

Figure 3 Particle size distribution of the optimized DPI

Aerosol Characteristics by Cascade Impaction

The aerodynamic behaviour of the particles was studied by cascade impaction. Powdered particles was administered to the laboratory animals through inhalation by in-house apparatus. The delivery port of the inhalation apparatus was interfaced with a MOUDI 100 cascade impactor (10-stage impactor, MOUDI 100NRl, TSI Inc., USA). The 10 mg of powder samples (n=3) were fluidized at negative airflow rate of 30L/min. The deposited powder at different stages of the cascade impactor was weighed using a five-digit balance (Mettler, USA). The effecive cut of diameters (ECD) of stages from 0 to 10 were 18, 10,5.6, 3.2, 1.8, 1, 0.56, 0.32, 0.18, 0.1 and 0.056 µm respectively. The Mass Median Aerodynamic Diameter (MMAD) and the Fine Particle Fraction (FPF) were estimated from the amount (%) of the powder deposited on each stage. MMAD was

calculated as 50 % cumulative undersize from the best fit log-probability graph of cumulatve % frequency undersize against ECD. Geometric standard Deviation (GSD) was calculated from the ratio 82% to 50% undersize. The relative Fine Particle Fraction (FPF) was calculated from the percent cumulative mass undersize.

Cascade impaction estimated a mass median aerodynamic diameter of 2.85µm and a geometric standard deviation of 1.8 (Figure.7 A), with a fine particle fraction (FPF<3.2) of 58.33±4.74% (Figure 7 B), n=3. These findings show that the powder is suitable for deep lung deposition, but also suggest that further optimization is needed to improve the aerosol characteristics.

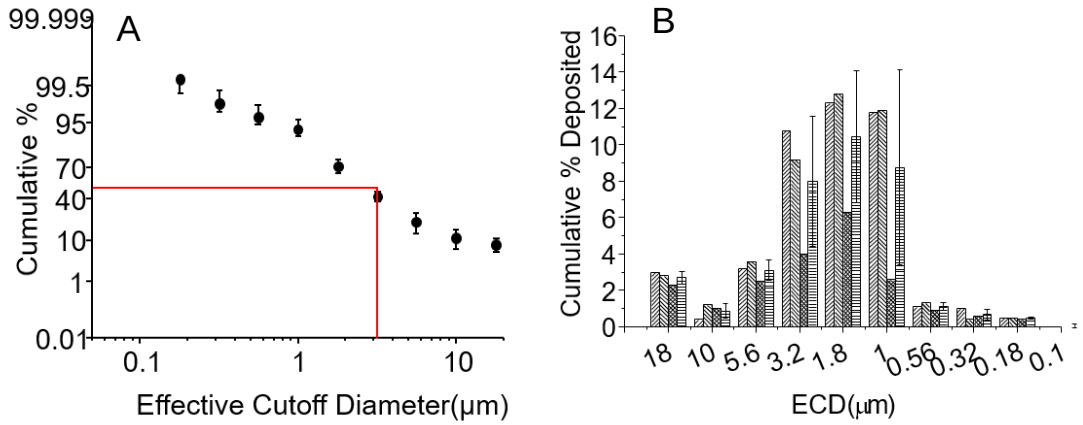

Figure 4 (A): MMAD calculated as 50% undersize and GSD at the 16th and 84th percentile from data on cumulative % deposition at different ECDs. (*B*): The FPF (<3.2µm; stages 4-10) was 58.33±4.74%.

These properties indicate that the powder is suitable for deep lung deposition of particles. β-cyclodextrin, the principal constituent of the particles is expected to dissolve rapidly on deposition at the well-hydrated airway and lung epithelium, especially in the presence of lung surfactant. The DNA polyplex is thus likely to be made available to epithelial cells rapidly, as phagocytes would have a very small-time window to take up deposited particles.

Table 1 summarizes the characteristics of the powder. Results obtained by various techniques are discussed in subsequent Sections.

Table 1: Physical characteristics of prepared particles.

Parameters	**Value**
Bulk Density (g/cc)	0.0059
Tapped Density(g/cc)	0.014
Carr's Index	64
Angle of repose (degrees)	22°
Hausner's ratio	2.8
Median diameter by SEM (µm)	1.5µm
Volume-average diameter by DLS (µm)	10µm
Mass Median Aerodynamic Diameter, MMAD (µm)	2.85
Geometric Standard Deviation (GSD) from MMAD (µm)	1.84
Fine Particle Fraction< 3.2 µm, FPF (%)	92.2

Efficacy Studies—Transient Transfection *In Vitro* and *In Vivo*

Reporter Gene Expression Following In Vitro Transfection—Fluorescence and Confocal Microscopy and Flow Cytometry

A549 cells were seeded on 6-well black plates (SPL Life Sciences) at a density of 10^5 cells per well 24 hours before transfection. Polyplexes of bPEI/DNA were prepared by incubating 1µg of pDsRedExpressN-1 for 30 minutes at room temperature with appropriate amounts of bPEI at N/P ratios of 1:2. The final volume was adjusted to 3ml with serum-containing RPMI medium, and different quantities of bPEI (N/P of 1:2) weight per 1µg of pDNA were added. The bPEI/DNA complexes were added to a six-well black plate and incubated at 37°C for 6h, 12h, 18h,24h.33h, and 48h.To ensure reproducibility, all of the tests were carried out in quadruplets. Cells were rinsed with sterile 1X PBS buffer pH 7.4 and cells were incubated with DAPI solution of 0.1 µg/ml and

Lysotracker Red for 10 min. The cells were washed with PBS at pH 7.4 and observed under a fluorescence microscope and the confocal microscope.

We observed that after introducing DPI particles to cells, IFN-γ protein was secreted into the culture medium (Figure 8). The expression profile was biphasic in time, with two distinct peaks at 6- and 24-hours post exposure. Expression was also dose- and time-dependent. Thus, transfection of 10^5 cells with either 1 ng or 1.5 ng of DNA resulted in a concentration difference of 10 pg/ml of the protein at 6- and 24-hours post exposure. Similarly, the higher dose resulted in longer prevalence of detectable concentrations in the culture medium. Cells transfected with 1.5 ng DNA continued to secrete detectable IFN-γ for about 8 hours longer than was observed with cells receiving 1 ng DNA.

Flow cytometry of cells transfected with gfp-Ifng revealed that the foreign protein's intracellular concentrations peaked at 12 hours after transfection (Figure 8B). The mean fluorescence intensity (MFI) corresponding to GFP rose from less than 20 arbitrary units at zero time to more than 60 units at the peak at 12h.

Confocal microscopy at different time intervals revealed that fluorescence could be observed starting at 6 hours after exposure to particles bearing gfp-Ifng (Figure 8 C, D). At 48h post transfection, GFP co-localized with Lysotracker Red® dye. This observation again confirmed that the expressed chimeric protein was trafficked to lysosomes for degradation. Differences were observed in the expression and degradation of the native and chimeric proteins, as was expected. These results can also be interpreted as *in vitro* ‘pharmacokinetics’ of IFN-γ secretion by cultured cells transfected using the DPI. Epithelial cells do not normally produce IFN-γ. Transfection with the formulation gave rise to a concentration-time profile of the cytokine, suggestive of the profile that may be obtained in animals receiving inhalations. However, *in vitro* transfection efficiency is likely to be significantly higher than the efficiency achievable *in vivo*.

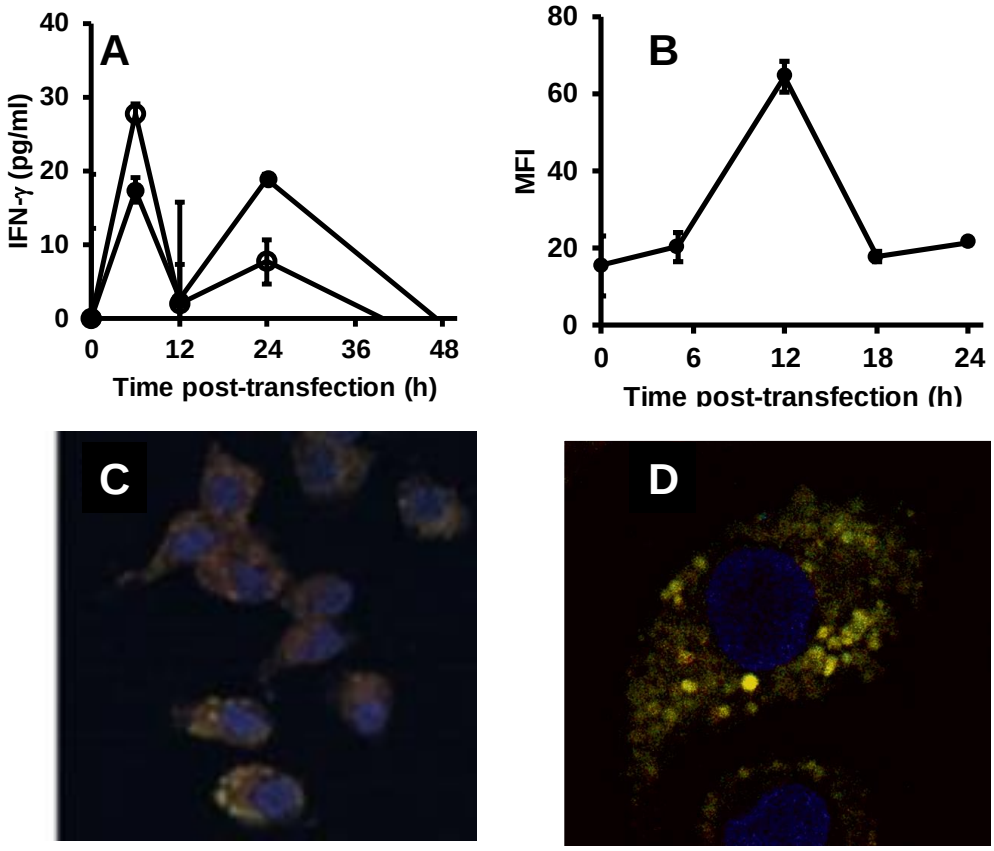

Figure 8 Transient gene expression *in vitro*. (A): IFN-γ secreted in culture supernatant by 3×10^5 A549 alveolar epithelial cells transfected with about 1 ng (*filled symbols*) or 1.5 ng (*open symbols*) of plasmid DNA. Means ± SD of three replicates is plotted. (B): A chimeric GFP-IFN-γ protein was not secreted but could be detected by flow cytometry. Means ± SD of three replicates is plotted. (C): The green fluorescence attributable to the chimeric protein co-localized with Lysotracker Red at 48h post-transfection to generate yellow fluorescence. (D): Detail of (C).

Preclinical Pharmacokinetics and Drug Disposition in Mice

All experiments were carried out after receiving approval from the Institutional Animal Ethics Committee (IAEC). For in-vivo imaging, six male Swiss mice weighing between 15-20g were randomly allocated to control and treatment groups (*N*=3/group). DPI were administered using the in-house inhalation apparatus. Animals were restrained for 30 seconds for nose-only exposure to

DPI aerosols using the in-house apparatus standardized in the lab. At time intervals of 6h, 12h, 18h, and 31h, mice were anesthetized with isoflurane and in-vivo imaging carried out as per the manufacturer's instructions (IVIS Spectrum, Perkin-Elmer, USA).

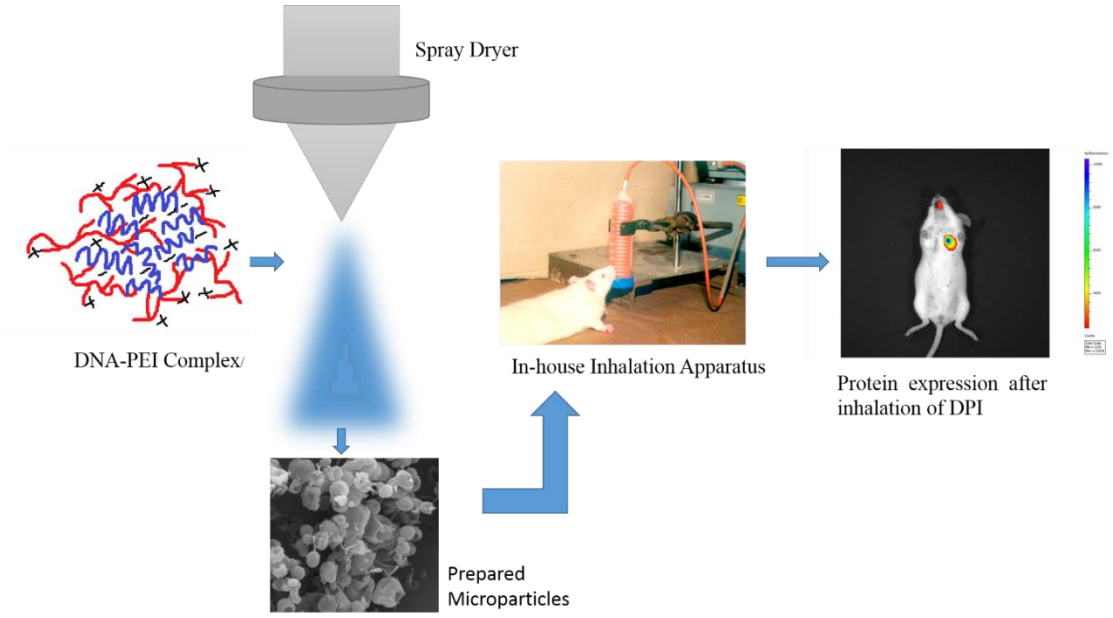

Figure 9. Schematic representation depicting the method of preparation and administering the DPI to mice.

In-vivo, mice inhaled particles containing a plasmid DNA with RFP under the CMV promoter. Imaging studies indicated that RFP expression was detectable as early as 6 hours post-inhalation, peaked at 24 hours, and substantially decreased by 48 hours (Figure 10). This confirmed the transient nature of the gene expression induced by the DPI formulation. This study also highlighted a limitation of dry powder inhalations in general, but especially applicable to the use of DPI for *in vivo* transfection. One mouse out of three that received inhalations of a plasmid expressing RFP showed the fluorescence signal in only one lung (10G). This observation might be attributed either due to deposition of the inhaled powder in only the left lung, or failure of transfection in the right lung even though the powder may have deposited in both lungs. Non-uniform and idiosyncratic deposition of inhaled powder in the airways and lungs is therefore the likely explanation for this observation. If this happened to a patient with tuberculosis in the lungs, it would be the equivalent

of missing a dosage. This is also why our medicine is marketed as a pulmonary TB adjunct or add-on therapy(15).

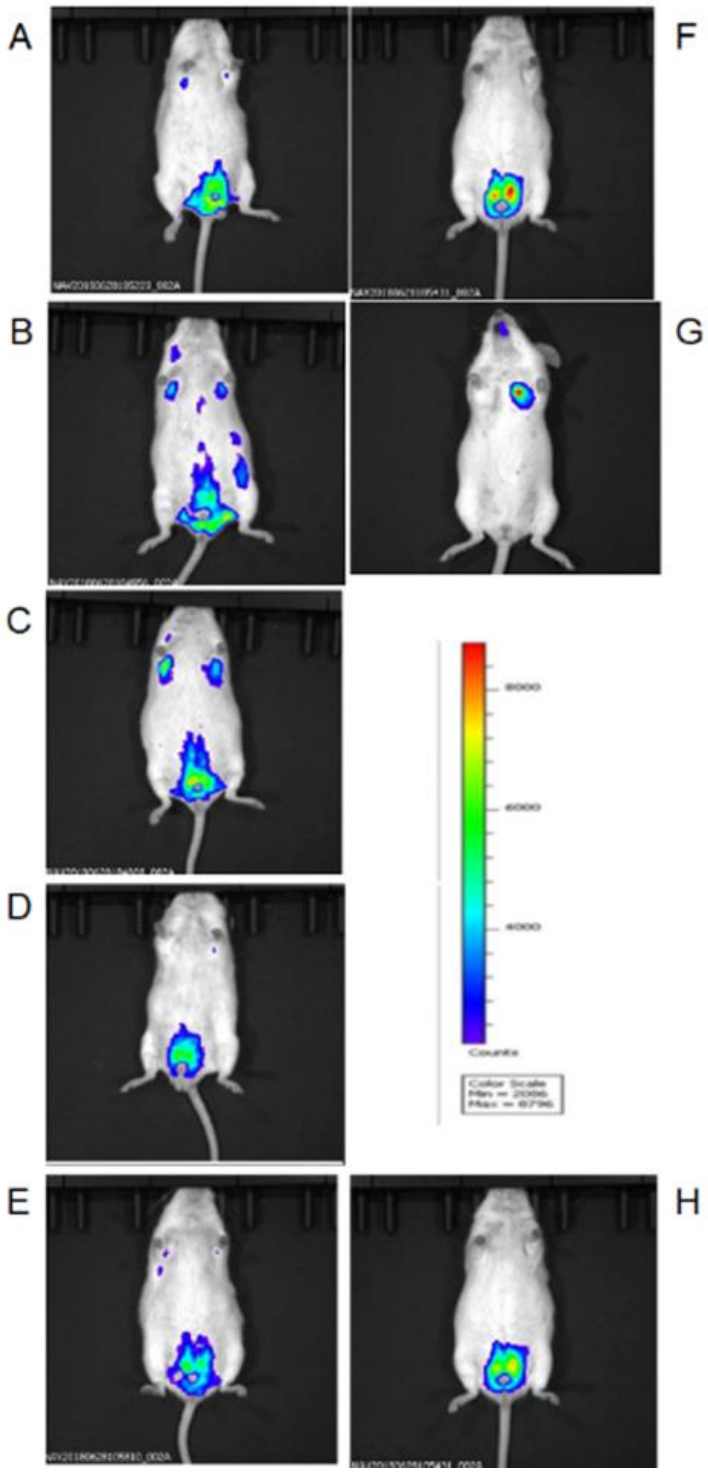

Fig 10. In- vivo **imaging by IVIS spectrum at different time intervals of control and treated mice after inhalation of RFP-expressing plasmid. (A-E): 6; 12, 18, 24 and 31h after inhalation. (F): Animal that received control particles without DNA at 6h. (G): One animal showed fluorescence in the nose and mouth, but only in the left lung at 12 hours (*corresponding to the animal shown in Panel* B). (H): Control animal, the same as shown in Panel F, at 6h**

Immunohistochemistry of mice lungs after DPI Inhalation

Mice were sacrificed at different timepoints after DPI inhalation, and lungs were harvested from these mice. Lung tissue samples were stored in 10% formalin and embedded in paraffin following dehydration. Tissue sections with a thickness of 5 m were prepared using a microtome (Leica, RM225), deparaffinized with xylene, and rehydrated in graded alcohol according to standard procedure. Citrate buffer was used for antigen retrieval (pH 6.0). After that, the sections were permeabilized for 15 minutes with 0.5 percent Triton X-100. Primary antibodies against LC-3 (autophagosomes) and LAMP-1 (lysosomes) were incubated overnight at 4°C on permeabilized sections. The tissue sections were washed three times with PBS having 1% BSA. The nuclei in the sections were stained with DAPI and the sections were washed three times. The tissue sections were mounted with antifade (ProLongTM Gold antifade reagent, Invitrogen by Thermo Fisher Scientific) and observed under a confocal microscope (Carl Zeiss LSM 510 Meta) at 63× magnification using 1.40 oil immersion objective.

Colocalization analysis was done using WCIF image J localization plugin. Qualitative analysis was done by highlighting the overlapped pixels. The quantitative analysis was done by finding the mean fluorescence intensity of colocalized points (Histogram analysis), positive product of differences from the mean (PDM) values, % colocalization and correlation coefficients were calculated.

The PDM value for the pixel was calculated as:

PDM= (red intensity -mean red intensity) × (green intensity -mean green intensity)

Figure 11 demonstrates that LC3-II and LAMP-1 expression was low in the lungs of mice that had not been treated and those that had received inhalations 7 hours prior to harvesting the lungs. The expression of these markers rose noticeably after 18 hours. At 48 hours after inhalation, the two markers co-localized.

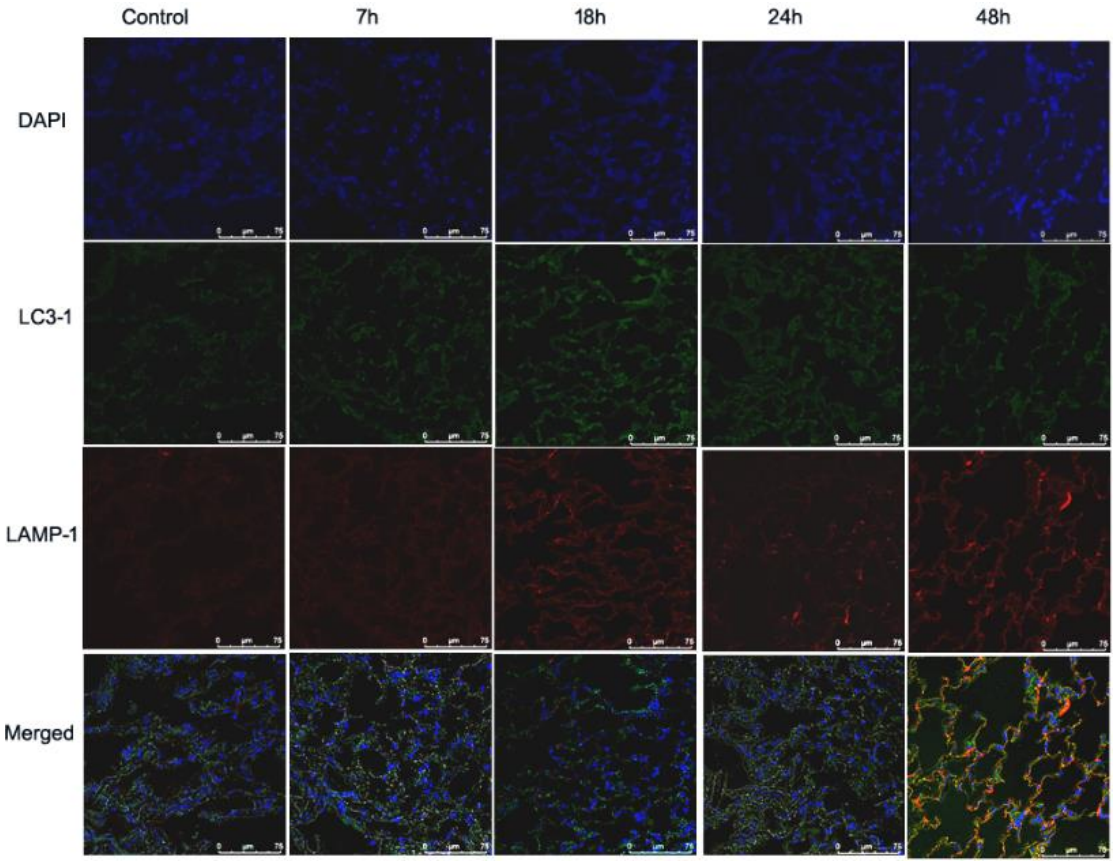

Figure 11. Representative lung sections of mice infected with MTB 21 days before dosing with inhalations of the plasmid bearing the IFN-γ gene indicate progressive induction of autophagy as surrogate pharmacodynamic response to transient transfection of the lung. The response was observed to initiate at 18h and was maximal at the last time-point studied.

The current finding shows that a single inhaled dosage of IFN-γ induces autophagy in the lungs cells.

Pharmacokinetics of IFN-γ in mice

Inhalation of the DPI carrying the plasmid bearing the mouse gene for IFN-γ was given to healthy mice (at CDRI). IFN-γ was quantified using an ELISA carried out in the BAL fluid recovered from the animals at the time of terminal sacrifice. Additionally, we estimated IFN-γ in lung homogenates and blood serum of mice at various time periods. The pharmacokinetic modelling software PK Solver 2.0 was used to fit the data to a one-compartment pharmacokinetic model with a temporal lag following extravascular administration. This was used to investigate if the results could help us understand the rates and extents of secretion of the cytokine. This exercise was an attempt to correlate a surrogate pharmacodynamic response (induction of autophagy, Figure 11) with prevailing concentration of an immune effector/ host defense molecule.

Encouragingly, quantifiable amounts of IFN-γ could be recovered from the animals by BAL. This observation indicates that sufficient transfection efficiency was achieved *in vivo* with the gene of interest. Quantitative comparison or *in vitro-in vivo* correlation (IVIC) between results presented in case of cultured lung epithelial cells were not attempted, because in the intact animal, a variety of cell populations are present that can express IFN-γ. A small stimulus with exogenously administered IFN-γ can potentially amplify the secretion of this cytokine by other cell types. Thus, *in vivo* results were interpreted as stand-alone observations, with the whole amounts of IFN-γ arising from the sum of the amounts expressed by the transfected plasmid, plus the amounts elicited from stimulated, endogenous cell populations.

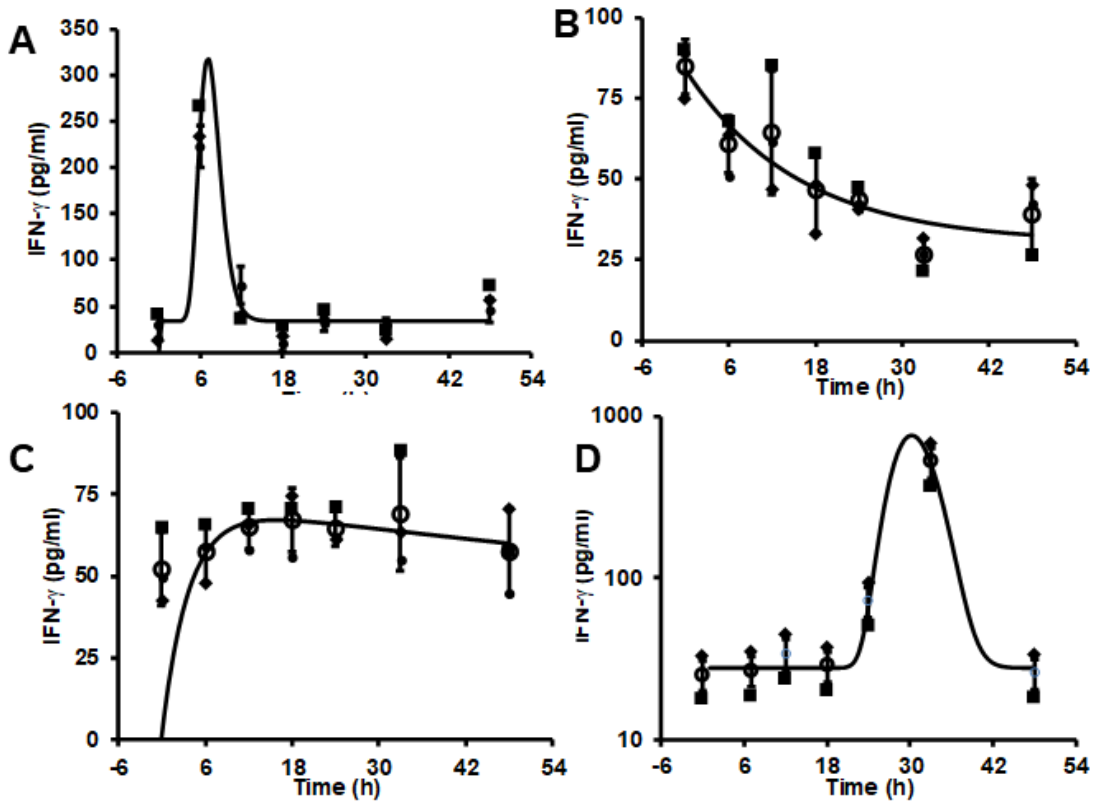

Figure12. Pharmacokinetics of IFN-γ in (*N*=3) uninfected (A-C) and (*N*=2) infected mice (D). Scatter points show values from individual animals, points with error bars indicate mean±SEM and solid lines depict model-fitted values of concentrations of IFN-γ as determined by ELISA in: BAL fluid (A), lung homogenate (B) and blood plasma (C) of uninfected mice; (D): The concentration-time profile in the BAL fluid of infected mice showing drastic shift in C_{max} and T_{max}.

Figure 12 illustrates the results obtained in respect of healthy, uninfected mice. Following a single inhalation dose of 5ng plasmid DNA, secretion of IFN-γ towards the luminal side of the airways and lungs was detectable in BAL. Concentrations in blood serum and lung tissue homogenate retrieved at these time points could also be established. In the BAL fluid, IFN-γ peaked around 8 hours after inhalation, and was detectable in the lung homogenate practically immediately after inhalation. In the BAL fluid, the half-life (t1/2) was around 3 hours. We found significantly large amounts in blood plasma, which were similar to those seen in BAL fluid at the earliest time point.

The BAL fluid pharmacokinetics of IFN-γ reflect single-dose preclinical pharmacokinetics in a tissue compartment that is rarely investigated in clinical trials.

Conclusion and Future Directions

This research demonstrated the feasibility of using a DPI formulation to deliver the IFN-γ gene for transient transfection in the lungs, offering a promising approach for lung-targeted gene therapy. The findings suggest that this prototype product is ready for detailed preclinical investigation of its safety and efficacy, particularly as an adjunct therapy to prevent severe immunopathology in respiratory diseases.

Application of IFN-γ dry powder inhalation (DPI) formulation

The dry powder inhalation (DPI) formulation developed for the delivery of interferon-gamma (IFN-γ) gene holds promise for its unique capability to deliver gene therapy directly to the lungs and opens up several potential therapeutic applications in respiratory diseases:

1. Lung Cancer: The DPI could be adapted to deliver genes that either suppress tumor growth or enhance immune response against lung cancer cells. For example, genes encoding tumor suppressor proteins, pro-apoptotic factors, or cytokines that stimulate anti-tumor immunity could be delivered directly to the tumor site, enhancing the efficacy of treatment while minimizing systemic side effects. The DPI could be used in combination with existing immunotherapies, such as immune checkpoint inhibitors, to potentiate the immune response within the tumor microenvironment. By delivering immune-activating genes locally, the DPI could help overcome tumor immune evasion mechanisms.
2. Chronic Obstructive Pulmonary Disease (COPD): Anti-inflammatory Gene Delivery: COPD is characterized by chronic inflammation and tissue damage in the lungs. The DPI formulation could be used to deliver anti-inflammatory genes, such as those encoding interleukin-10 (IL-10) or other cytokines that modulate the immune response, directly to the lungs. This approach could help reduce inflammation and slow disease progression. Regenerative Therapy: Gene therapy aimed at promoting tissue repair and regeneration could be a novel approach to treating COPD. The DPI could deliver genes that stimulate the repair of damaged lung tissue or enhance the function of remaining healthy cells.

3. Other Respiratory Diseases: Cystic Fibrosis: The DPI could be utilized to deliver corrective genes to the lungs of patients with cystic fibrosis (CF), potentially restoring normal function to the defective CFTR protein. This could provide a less invasive and more efficient alternative to current gene therapy approaches for CF. Pulmonary Fibrosis: Gene therapy aimed at modulating fibrotic pathways could be delivered via the DPI to slow or reverse the progression of pulmonary fibrosis. This could include genes that inhibit fibroblast activation or promote the degradation of extracellular matrix components.

To fully realize the potential of the DPI formulation, preclinical development needs to be explored in the following direction:

1. Exploring the downstream effected of this therapy on genes: Downstream and upstream effect of the DPI induced gene therapy should be carefully studied to evaluate safety/toxicity issues.
2. Conduct of Essential Safety Pharmacology and Toxicity studies in rodents and non-rodents.
3. Preclinical Efficacy as Adjunctive Treatment with Conventional Therapies: The DPI could be studied as an adjunctive therapy alongside conventional treatments such as chemotherapy, radiotherapy, or standard anti-inflammatory drugs. This could involve using the DPI to deliver genes that mitigate the side effects of these treatments or enhance their efficacy.
4. Preclinical Efficacy in Animal Models of Other Diseases: Additional preclinical studies in relevant animal models could help establish the efficacy and safety of the DPI formulation for various diseases. These studies would be critical for optimizing dosing regimens,
5. Pharmacokinetics and Biodistribution: Studies to determine the pharmacokinetics (PK) and biodistribution (BD) of the DPI formulation in disease-specific models. These studies will assess how the formulation is absorbed, distributed, metabolized, and excreted, as well as how long the gene expression lasts in the lung tissue.

6. Proof-of-Concept Studies: Demonstrating the efficacy of the DPI in animal models of target diseases (e.g., lung cancer, COPD, tuberculosis) is critical. These studies will help establish the therapeutic potential and optimal dosing regimen.
7. Manufacturing Scale-Up: Scaling up the manufacturing process to produce clinical-grade DPI formulations must adhere to GMP standards. This involves optimizing the production process to ensure consistency, quality, and safety.
8. Quality Control and Assurance: Rigorous quality control measures must be established to monitor the physical and chemical properties of the DPI, including particle size, gene integrity, and sterility.
9. Studies required for Regulatory clearance from the review committee on genetic manipulation (RCGM) of the DBT

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Signed

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